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Detection, monitoring & clonal characterisation of human cytomegalovirus specific $CD8^+$ T cells in haematopoietic stem cell transplant patients

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I, Sandra Giest, confirm that the work presented in this thesis is my own.
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"The history of science - by far the most successful claim to knowledge accessible to humans - teaches that the most we can hope for is successive improvement in our understanding, learning from our mistakes..., but with the proviso that absolute certainty will always elude us."

Carl Sagan

DEDICATION

Dieses Werk ist den drei für mich wichtigsten Menschen gewidmet: Meinen Eltern, Klaus und Renate Nowack, die alles daran setzten, mir den Zugang zu Literatur und Studium zu ermöglichen und mich in meinen Vorhaben jederzeit liebevoll unterstützt haben; sowie meinem geliebten Ehemann, Sascha, der mir mit Liebe, Zuversicht, unglaublicher Willensstärke und Vertrauen zur Seite steht und mir nach der ungeplanten Unterbrechung half, Kraft zu finden, dieses Projekt wieder aufzunehmen und zu vollenden.

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ABSTRACT

The human cytomegalovirus (CMV) can cause significant morbidity and mortality in haematopoietic stem cell transplant (HSCT) patients. The immunosuppressed state of the hosts facilitates dissemination of the virus and disease. In contrast, $CD8^+$ T cells in healthy individuals control viral dissemination and maintain a balance between antiviral host defence and replication of the virus that leads to viral latency with sporadic, harmless reactivations.

Current pharmacological intervention in the HSCT setting is associated with significant toxicity and is counteracted by the occurrence of resistant viral strains. Alternative approaches, such as adoptive therapy of CMV specific $CD8^+$ T cells are of great interest in the field. So far, levels of such cells correlating with protection against CMV disease in patients were only shown for cells targeting two different viral epitopes.

The project described in this thesis investigates $CD8^+$ T cell responses to several common CMV targets presented by different human leukocyte antigens in the HSCT setting. Results demonstrate significant differences between the numbers of different CMV specific $CD8^+$ T cells that, in the presence of $CD4^+$ T cell help, inversely correlate with the ability to detect CMV reactivation. Findings also demonstrate significant differences in the diversity of T cell receptors (TCRs) used by the different CMV specific $CD8^+$ T cells isolated from HSCT patients.

These findings are clinically relevant in that the quantity of cells shown to correlate with protection against CMV could be used as a marker for monitoring patients' immune status towards CMV. This may aid clinical decision making to limit pharmacological intervention to those patients at highest risk for the development of CMV disease. It may also aid the monitoring of the effectiveness of adoptive therapy trials. Therapeutic use of cells with high TCR diversity may be advantageous over other cells in that they may impede the development of CMV immune escape in patients.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

The following abbreviations were used throughout this thesis, where each citation (with the exception of A, C, G, T, v/v and w/v) was spelled in full on first appearance. Most common general abbreviations of units or names used are not listed here.

A	adenine
AA	aplastic anaemia
Ab	antibody
ACS	American Chemical Society
ACV	aciclovir
AIDS	acquired immune deficiency syndrome
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANRI	Anthony Nolan Research Institute
APC	antigen presenting cell(s) or allophycocyanin
APS	ammonium persulphate
ATG	antithymocyte globulin
ATP	adenosine-5'-triphosphate
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine
BCNU	1,3-bis (2-chloroethyl)-1-nitroso-urea
BD	Becton Dickinson
$ß_2m$	$ß_2$ microglobulin
BFA	brefeldin A
BiP	immunoglobulin-binding protein
BM	bone marrow
BMT	bone marrow transplantation

bp	base pairs
Bromophenol blue	3',3",5',5"-tetrabromophenolsulfonphthalein
BSA	bovine serum albumin
bsp	BirA substrate peptide
C	constant or cytosine
CA	carbonic anhydrase
CB	cord blood
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementarity-determining regions
CDV	cidofovir
CFDASE	5,6-carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
cj	coding joint
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CpG	cytosine and guanine separated by a phosphate
CSA	cyclosporine A
DB	dense bodies
DC	dendritic cell(s)
DGGE	denaturing gradient gel electrophoresis
DISC	death inducing signalling complex
DLI	donor lymphocyte infusion
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid

dNTP	deoxynucleotide-triphosphate
DTT	dithiotreitol
E	early CMV genes or their products
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot
EP	erythropoietic protoporphyria
EPE	extracorporeal photopheresis
ER	endoplasmatic reticulum
ERAAP	ER associated aminopeptidase
ET	energy transfer
FACS	fluorescent-activated cell sorting
FADD	Fas-associated protein with Death Domain
FAM	6-carboxyfluorescein
FasL	Fas ligand
FCN	foscarnet
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FPLC	Fast Protein Liquid Chromatography
FRET	fluorescence resonance energy transfer
FSC	forward scatter

G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS	IFNγ activated sequences
gC	glycoprotein complex I-III of CMV
G-CSF	granulocyte colony stimulating factor
GCV	ganciclovir
GR	glucocorticoid receptor
GRE	glucocorticoid response elements
GvH	graft *versus* host
(a/c) GvHD	(acute/chronic) graft *versus* host disease
H&E	haematoxylin and eosin
HD	Hodgkin's disease
HEV	high endothelial venules
HHV	human herpes virus
HIS	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HSC	haematopoietic stem cell(s)
HSCT	haematopoietic stem cell transplantation
HSPG	heparan sulfate proteoglycans
hTERT	human telomerase reverse transcriptase
ICAM	intercellular adhesion molecule
ICC	intracellular cytokine
ICS	intracellular staining

IE	immediate-early CMV genes or their products
IFN(γ)	interferon (gamma)
Ig	Immunoglobulin
IL	interleukin
IPTG	isopropyl ß-D-1-thiogalactopyranoside
IRL	internal repeat long of the CMV genome
IRP	immune-risk phenotype
IRS	internal repeat short of the CMV genome
ITAM	immunoreceptor tyrosine-based activation motifs
J	joining
Jak-STAT	janus kinases- signal transducers and activators of transcription
JOE	6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein
kb	kilobase
kDa	kiloDalton
KIR	killer-immunoglobulin-like receptor
KLRG1	killer cell lectin-like receptor G-1
L	late CMV genes or their products
Lck	leukocyte-specific protein tyrosine kinase
LDA	limiting dilution assay
LFA	lymphocyte function-associated antigen
LPA	linear polyacrylamide
mAb	monoclonal antibody
MAdCAM	mucosal addressin cell adhesion molecule
MBP	myelin basic protein
MCS	multiple cloning site
MDS	myelodysplastic syndrome

MHC	major histocompatibility complex
MIEP	major immediate-early promoter
MM	multiple myeloma or (HLA) mismatched
MMF	mycophenolate mofetil
M-MLV	Moloney Murine Leukaemia Virus
mRNA	messenger ribonucleic acid
MTX	methotrexate
MWCO	molecular weight cut off
NBT	nitroblue tetrazolium chloride
NF-κB	nuclear factor-kappa B
NHL	non-Hodgkin's disease
NIEP	non-infectious enveloped particles
Ni-NTA	nickel- nitrilotriacetic acid
NK cell	natural killer cell
OD	optical density
OH	hydroxyl
ORF	open reading frames
OVA	Ovalbumin
PBMC	peripheral blood stem cell(s)
PBS	phosphate buffered saline
PBSC	peripheral blood stem cell(s)
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
pfu	plaque-forming units

PHA	phytohaemagglutinin
PMSF	phenylmethylsulphoxide
PMT	photomultiplier tubes
pp	phosphoprotein
qPCR	quantitative PCR
RAG	recombination activating gene
RFH	Royal Free Hospital
RIC	reduced intensity conditioning
RNA	ribonucleic acid
ROX	5-carboxy-X-rhodamine
RPMI (1640)	cell culture medium developed at the Roswell Park Memorial Institute
RT	reverse transcription
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFU	spot-forming unit
SH2	src-homology type 2 domain
sj	signal joint
SSC	side scatter
SSO	sequence-specific oligonucleotide
SSP	sequence-specific primer
T	thymine
TAP	transporter associated with antigen presentation
TAMRA	N,N,N',N'-tetramethyl-5-carboxyrhodamine
TBE	Tris-Borate-EDTA
TBI	total body irradiation
TBS	Tris buffered saline

TCD	T cell depletion
T_{CM}	central memory T cells
TCR	T cell receptor
T_{EM}	effector memory T cells
TEMED	tetramethylethylenediamine
TfR	transferrin receptor
TLR	Toll-like receptor
TNF(α)	tumour necrosis factor (alpha)
TOLEDO-F	fibroblast adapted TOLEDO laboratory CMV strain
TREC	TCR excision circle
Tris	trishydroxymethylaminomethane
TRL	terminal repeat long of the CMV genome
TRS	terminal repeat short of the CMV genome
UL	unique long region of the CMV genome
UAdM	Universidad Autónoma de Madrid
UFdP	Universidade Federal do Paraná
US	unique short region of the CMV genome
V	variable
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

CHAPTER 1 INTRODUCTION

The English scientist Dr. James Ephraim Lovelock once said *"An inefficient virus kills its host. A clever virus stays with it"*. The human cytomegalovirus (CMV) is an ancient virus, which is well adapted to its host. It has evolved strategies to avoid its elimination and can exist in a latent form in individuals. Nevertheless it is not adapted to artificial circumstances brought by recent medical advances and can cause significant morbidity and, in some cases, mortality in patients who received immunosuppressive conditioning prior to haematopoietic stem cell transplantation (HSCT).

The project described in this thesis investigates immune responses to CMV in the HSCT setting, where clinical intervention is needed to restore the balance between virus and host. This chapter will introduce background information on some features of CMV, the human immune response to the virus and particularly its cellular compartment, immune evasion strategies evolved by CMV, HSCT and the role of CMV in the transplant setting as well as other information directly relevant to this investigation. Attention is focused on CMV specific $CD8^+$ T cells that may be used for adoptive immunotherapy and whose monitoring may be clinically advantageous during periods of immunodeficiency in HSCT patients. This chapter will also explain the choice of $CD8^+$ T cells that were studied within this project.

1-1 Cytomegalovirus infection

The human Cytomegalovirus is also called human herpesvirus 5 (HHV-5). It is a ubiquitous ß-herpesvirus (genus Cytomegalovirus (CMV), family Herpesviridae, subfamily Betaherpesvirinae) with a diameter of about 200 nm. It is a deoxyribonucleic acid (DNA) virus with the typical herpes virion structure as illustrated in Figure 1-1.

Figure 1-1 Structure of Cytomegalovirus

Left: model of CMV virion adapted with permission from Dr. M Reschke (http://www.biografix.de); right: Electron microscopic image of a herpes virion (c: capsid, t: tegument; e: lipid envelope; bar = 100 nm) adapted from (McGeoch *et al.*, 2006) with permission from the author.

Its double stranded DNA ranks amongst the largest of all DNA virus genomes containing more than 200 open reading frames (Chee *et al.*, 1990). The genome size varies in different strains. The low passage strain Merlin is 236 kilobase (kb) in size (Dolan *et al.*, 2004) whereas high passage strains have smaller genomes with AD169 and Towne containing 230 kb (Murphy *et al.*, 2003). The genome structure consists of two separate regions, the unique short and unique long sequences (US and UL respectively), which are flanked by terminal and internal repeats. In some laboratory strains repeats consist of the terminal repeat long (TRL), the terminal repeat short (TRS) and the two internal, inverted repeats (IRL and IRS), which allow viral progeny to contain four isomers, each differing in the orientation of the UL and US components to one another (Kilpatrick and Huang, 1977). Clinical isolates contain an additional DNA segment (UL133 - 151) and lack an IRL repeat (Murphy *et al.*, 2003). The CMV DNA is surrounded by a capsid consisting of 162 capsomeres (Wright *et al.*, 1964) arranged in an icosahedral structure (a polyhedron having 20 faces, usually regular: with equilateral triangles as faces). This nucleocapsid is surrounded by the tegument composed of phosphoproteins, which is enclosed by a host derived lipid bi-layer envelope spiked with viral proteins that are usually glycosylated and can be categorised into three families, the glycoprotein complexes (gC) I, gCII and gCIII. The envelope is acquired during the life cycle of the virus as illustrated in Figure 1-2.

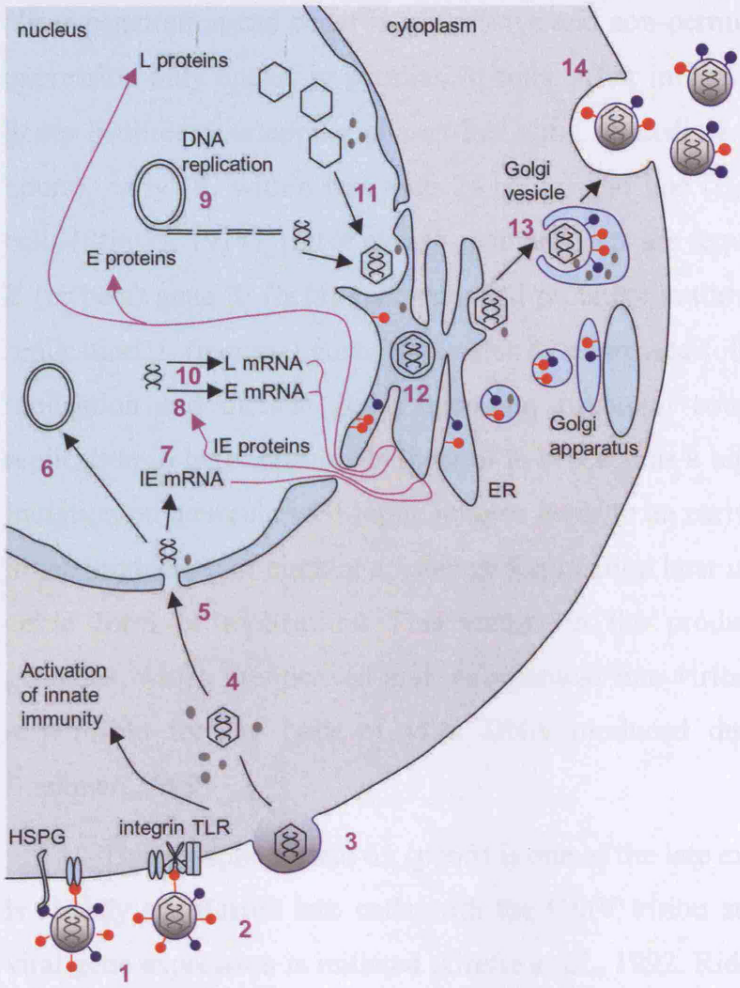


Figure 1-2 CMV lytic cycle

CMV attaches by tethering (1) to cell surface heparan sulfate proteoglycans (HSPG) via its glycoproteins (Compton *et al.*, 1993) resulting in clustering with other receptors of which suggested candidates are aminopeptidase N (CD13) (Soderberg *et al.*, 1993) and epidermal growth factor receptor (EGFR) (Wang *et al.*, 2003). Post attachment integrin interaction (Feire *et al.*, 2004) (2) helps the viral envelope to fuse with the plasma membrane of the cell (3). The binding of CMV also triggers an inflammatory response via Toll-like receptor (TLR) 2 and CD14 (Compton *et al.*, 2003). The viral capsid containing the genome and tegument proteins enters the cytoplasm. The capsid then travels along microtubules (4) towards the nucleus, where uncoated viral DNA enters through nuclear pores (5) and circularises (6). Viral DNA is transcribed in the nucleus, messenger ribonucleic acid (mRNA) is transported to the cytoplasm for translation of proteins that then travel back to the nucleus or are delivered from the endoplasmatic reticulum (ER) to the Golgi apparatus. Transcription occurs in a cascade with immediate-early (IE) transcripts (7) triggering transcription of early (E) genes (8), which amongst other functions participate in viral DNA replication (9) and trigger transcription of late (L) genes (10), which amongst other functions provide capsid proteins. Encapsidation of DNA and assembly of nucleocapsids in the nucleus (11) are followed by primary envelopment in the perinuclear space (12), which expands into the ER. The temporary envelope acquired during budding into the inner nuclear envelope is lost by fusion with the outer nuclear envelope. Naked capsid subsequently progresses through the cytoplasm and buds into Golgi vesicles laden with viral proteins. This process provides the developing virion with tegument proteins (●), an envelope (--) and surface glycoproteins (● ●) (13). The Golgi vesicle delivers the new virion to the cell surface, where it fuses with the cell membrane releasing new infectious virus (14). This figure was created based on information from (Boehme, 2006, Mocarski, 2006, Mocarski, 2007).

Virus penetration can occur in permissive and non-permissive cells alike but viral gene expression only occurs in permissive cells. After infection, the viral genome expresses genes in three overlapping phases including immediate early (IE, within the first two hours), early (E, within less than 24 hours) and late (L, after 24 hours) genes in host cells (Stinski, 1978). IE (or alpha) gene products are required for the trans-activation of E (or beta) gene. E (beta) gene-encoded proteins, in turn, are necessary for viral DNA replication. L (gamma) genes are primarily expressed following the onset of viral DNA replication and include genes encoding structural components of the virion. DNA replication in herpesviruses is thought to proceed as a biphasic process. Origin-specific initiation on a circularized input genome leads to an early, theta mechanism that results in the production of circular episomes. Replication later undergoes a switch to a rolling-circle form of replication. This results in the production of linear concatemeric genomes, which are cleaved and incorporated into virions. This form of replication is responsible for the bulk of viral DNA produced during infection. (Lehman and Boehmer, 1999)

The phosphoprotein 65 (pp65) is one of the late expressed tegument proteins but is already transferred into cells with the CMV virion at the onset of infection before viral gene expression is initiated (Grefte *et al.*, 1992, Riddell *et al.*, 1991a). The protein is therefore widely used in the diagnosis of CMV infection and is thought to be one of the main targets of the $CD8^+$ T cell response against CMV, which will be described in more detail later.

CMV infected cells appear round and enlarged with intracellular inclusion bodies (Albrecht and Weller, 1980) as shown in Figure 1-3. These features were initially described as cytomegalic inclusion disease. The name cytomegalovirus was later used to reflect the cellular changes caused by the virus.

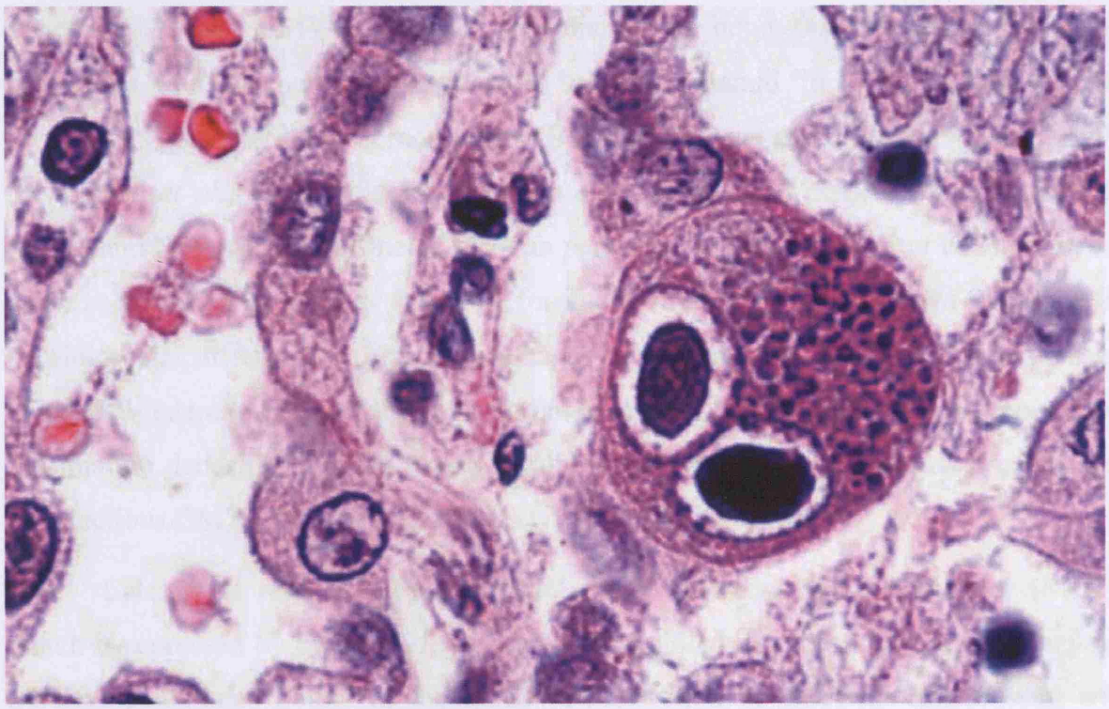


Figure 1-3 Characteristic appearance of CMV infected cells

Haematoxylin and eosin (H & E) stain of lung section (480x) adapted with permission from Dr. D. L. Wiedbrauk (http://www.microbelibrary.org). This stain demonstrates blue colouring of basophilic structures such as nucleic acids by haematoxylin and pink colouring of eosinophilic structures such as proteins by eosin (red blood cells are stained red). An enlarged CMV infected cell can be seen at the right part of the figure. The stain shows the formation of characteristic intranuclear inclusions (called owl's eyes) in the infected cell as well as capsids formed in the cytoplasm.

Cytomegaloviruses are highly species specific, which is indicative of a long co-evolution with its host. Human CMV productively infects a number of human cell types including fibroblasts, endothelial cells, epithelial cell and smooth muscle cells (Plachter *et al.*, 1996). After a lytic cycle (Figure 1-2), it usually establishes lifelong latency (Sissons *et al.*, 2002) during which CMV is kept firmly under control by persisting humoral and cell-mediated immune responses (Zanghellini *et al.*, 1999). Latency is a typical characteristic of all herpesviruses, during which the viral genome persists in specific host cells in the absence of any detectable production of infectious virus but with the ability to reactivate under specific stimuli. Along with infection of immune privileged sites such as retinal pigment epithelium (Scholz *et al.*, 2003) latency is one of a vast variety of immune evasion strategies (compare section 1-6 and 1-9.3) ensuring virus survival within the host. Host cells latently infected with CMV demonstrate altered expression of genes affecting functions such as immunity, cell growth, signalling and transcriptional regulation that may confer a survival advantage to the virus during latency or during the initial steps of virus reactivation (Slobedman and

Mocarski, 1999). CMV latency is established in bone marrow derived myeloid precursors and the virus persists as a circular plasmid in $CD34^+$ bone marrow progenitors, peripheral blood monocytes (Bolovan-Fritts *et al.*, 1999, Hahn *et al.*, 1998, Mendelson *et al.*, 1996, Taylor-Wiedeman *et al.*, 1991) and possibly endothelial cells (Jarvis and Nelson, 2002). Since CMV can persist in myeloid progenitor cells, these can serve as a renewable primary reservoir for latent virus. It has been shown that once the myeloid cells differentiate into macrophages they reactivate viral IE gene expression (Taylor-Wiedeman *et al.*, 1994). Differentiation of $CD34^+$ bone marrow progenitors into mature dendritic cells (DC) also results in reactivation of IE genes and production of infectious CMV (Reeves *et al.*, 2005).

Latency is regulated by the CMV major immediate-early promoter (MIEP), which is repressed by binding of cellular transcriptional repressors that recruit enzymes modifying histones bound to the MIEP. Reactivation of CMV is concomitant with chromatin remodelling of the MIEP into a transcriptionally active state (reviewed in (Sinclair and Sissons, 2006)). Reactivation of CMV from latency and replication of CMV can be triggered by an inflammatory environment induced by cytokines (Kline *et al.*, 1998) or allogeneic stimulation (Soderberg-Naucler *et al.*, 1997).

It results in production of infectious virions, which are shed from mucosal surfaces and ensure spread of the virus between different hosts. CMV can be transmitted from mother to foetus or from person to person by saliva, sexual contact, breast-feeding, blood transfusion or transplantation of solid organs or haematopoietic stem cells. The virus is present in 40 - 100 % of individuals depending on different socio-economic regions ranging from highly developed areas to developing countries (Krech, 1973). In Europe and the USA previous CMV infection can be demonstrated in up to 80 % of healthy individuals by persisting titres of CMV specific Immunoglobulin (Ig) G (Gershon AA, 1997, Ho, 1991). The equilibrium between viral immune escape strategies and effective immune responses in healthy hosts results in generally asymptomatic infection with periodic chronic low-grade reactivation linked to stress events (Docke *et al.*, 1994, Toro and Ossa, 1996). The host's cytotoxic T cell response acts efficiently to clear productively infected cells thereby maintaining viral latency in immunocompetent hosts. However, uncontrolled CMV replication in the absence of an effective immune response fosters viral dissemination resulting in disease.

Accordingly CMV is one of the leading infectious causes of birth defects and a major opportunistic pathogen in patients with acquired immune deficiency syndrome (AIDS) as well as in otherwise immunosuppressed individuals such as recipients of organ and haematopoietic stem cell transplantations (HSCT).

1-2 Haematopoietic stem cell transplantation (HSCT)

HSCT has been established as an important treatment modality for patients with haematological diseases. It is based on destruction or significant reduction of haematopoietic cells using cytotoxic drugs and/or total body irradiation (conditioning) followed by haematological reconstitution from donor haematopoietic stem cells (HSC).

Reconstitution of the T cell compartment occurs through two main pathways. In addition to thymus dependent generation of new T cells derived from stem cells, reconstitution can also occur through thymus independent expansion of mature T cells (Fry and Mackall, 2005). Although homeostatic proliferation of T cells is tightly regulated under normal conditions, under conditions of lymphopenia, cells can divide until the cellularity of the T cell compartment has been restored (Crooks *et al.*, 2006, Goldrath *et al.*, 2000).

With the exception of memory lymphocytes that can persist for several years, most mature blood cells are short lived. They develop from pluripotent stem cells that have the capacity for self-renewal and can give rise to multilineage progenitors. Multilineage progenitors in turn produce progenitor cells that are committed to produce blood cells of individual haematopoietic lineages. Therefore HSC can differentiate to all blood cell lineages (reviewed in (Orkin and Zon, 2008)). HSC and their capacity to renew lethally damaged blood cells were first experimentally explored after the first atomic bomb explosions in 1945 (reviewed in (Little and Storb, 2002)). E. D. Thomas pioneered the application of the results from early animal studies to the transfer of stem cells to cancer patients with malignant stem cells (which are quiescent and therefore insensitive to conventional chemotherapy) from the 1950s onwards (Thomas *et al.*, 1957).

Today the transplantation of HSC is used as a therapy for life threatening congenital or acquired disorders of the haematopoietic system including various malignant and non-malignant diseases. Many if not most of these transplantations are performed for haematologic and lymphoid cancers. HSC can be harvested from three sources. Traditionally, HSC are located in the bone marrow (BM) and can be harvested

from the centre of a bone, typically the pelvis. Since HSC circulate between BM and blood in a circadian pattern (Mendez-Ferrer *et al.*, 2008) and granulocyte colony stimulating factor (G-CSF) treatment can mobilise HSC into the blood stream, peripheral blood stem cells (PBSC) can also be directly harvested from withdrawn blood. Transplants with PBSC are more convenient and have replaced BM transplantation in many instances. Alternatively umbilical cord and placenta can be used to harvest cord blood (CB) stem cells. CB transplants were demonstrated to require less stringent HLA matching with low incidences of graft *versus* host disease (GvHD, this term will be explained in detail later) but they result in slower reconstitution and are often limited in pluripotent stem cell dose (Rocha and Gluckman, 2006, Wagner *et al.*, 2002). Therefore the use of additional grafts can be beneficial for successful engraftment (Barker *et al.*, 2005). HSC can be used for autologous (from the patient's own HSC that may be purged of residual diseased cells, for example by antibody treatment) or allogeneic (from another person's HSC) HSCT. (reviewed in (Copelan, 2006))

Allogeneic donors include related donors (including syngeneic donor, that is an identical twin) or unrelated volunteers from donor registries such as the Anthony Nolan Trust. The match of the major histocompatibility complex (MHC) (which is called Human leukocyte antigen (HLA) in human cells) in donor and recipient (histocompatibility) greatly influences the risk of the transplantation (Flomenberg *et al.*, 2004). As will be described in more detail in section 1-6, T cells recognise antigen in form of peptides presented on HLA molecules. HSCT recipient T cells may recognise foreign donor antigens and reject grafts. Donor T cells may recognise recipient antigens, which can result in graft *versus* tumour effects in leukaemic patients but also graft versus host disease. To keep the risk of an allogeneic HSCT at minimum, the HLA tissue types of donors and patients are matched as closely as possible. Therefore sensitive HLA typing of major HLA class I (HLA-A, HLA-B and HLA-C) and major HLA class II (HLA-DR, HLA-DQ and HLA-DP) is usually performed at the molecular level. HLA are polymorphic proteins. The loci encoding these major HLA have each up to several hundred variants in their DNA sequence, which are called HLA alleles. A series of these HLA alleles on a chromosome is called HLA haplotype of which each individual has two (passed from their mother and father respectively) that make up their HLA tissue type. Known HLA alleles are updated by the Anthony Nolan Research Institute in the IMGT/HLA database, which is part of the international ImMunoGeneTics (IMGT) project (http://imgt.cines.fr). Data on known HLA alleles are

integrated into software tools that allow assessment of histocompatibility between any of the HLA class I and II alleles (Elsner *et al.*, 2004).

However, GvHD or graft rejection also occurs in patients that receive HSCT from donors who (according to current standards) are fully HLA matched and can be caused by minor histocompatibility antigens. These are proteins other than the MHC, which are nevertheless also polymorphic and can therefore be different in patient and donor. Y chromosome encoded minor histocompatibility antigens are the cause for observations of higher incidences of GvHD in men receiving an HSCT from female donors than those receiving it from male donors (Vogt *et al.*, 2002). The potential exposure to fetal antigens during pregnancy may additionally increase the risk of GvHD in HSCT recipients receiving grafts from a female donor that had been pregnant previously. Polymorphic genes encoding killer-immunoglobulin-like receptor (KIR) ligands (Miller *et al.*, 2007) or others involved in immune responses and particularly cytokines can impact upon GvHD (Cavet *et al.*, 2001).

GvHD can occur following transfer of viable donor lymphocytes into immunocompromised or closely HLA-matched recipients and is commonest following allogeneic HSCT. It involves the attack of recipient tissue cells by donor derived T cells that recognise antigenic disparities between donor and recipient.

Acute GvHD (aGvHD) occurs within the first 100 days after transplantation. It is initiated (first phase) by the pre-transplant conditioning, which damages host tissues and results in activation of antigen presenting cells (APC) and their subsequent release of inflammatory cytokines (Xun *et al.*, 1994). These include interleukin 1 (IL1), IL6, interferon γ (IFNγ) and tumour necrosis factor α (TNFα), which can result in up-regulation of adhesion molecules and host major histocompatibility complex (MHC) antigens, thereby enhancing the recognition of host tissue by donor T cells. The pre-transplant conditioning additionally causes damage to endothelial and epithelial cells such as the intestinal mucosa (Eissner *et al.*, 1995), which enables bacterial products to translocate from the intestines, which in turn results in release of additional IL1 and TNFα (Nestel *et al.*, 1992).

This first phase of aGvHD is self-limited in autologous HSCT but in allogeneic HSCT it can be followed by a second phase, in which donor T cells, which are stimulated by the inflammatory environment, recognise alloantigens. This can be achieved via two different pathways. The direct pathway involves recipient antigen

presentation by recipient APC (Shlomchik *et al.*, 1999) whereas the indirect pathway involves recipient antigen presentation by donor APC (Matte *et al.*, 2004).

Alloantigen recognition, in turn, results in the third phase of aGvHD comprising T cell secretion of IL2 and IFNγ, which can result in the recruitment of more T cells, natural killer (NK) cells, monocytes and macrophages. Primed mononuclear cells finally secrete more IL1 and TNFα leading to a new inflammatory cycle (cytokine storm) and contributing directly to cellular damage or apoptosis. (reviewed in (Antin and Ferrara, 1992, Krenger *et al.*, 1997))

Chronic GvHD (cGvHD) is defined as occurring later than 100 days post HSCT either after acute GvHD or without previous aGvHD symptoms. At this time 100 % chimaerism of skin APC has been demonstrated suggesting that cGvHD involves indirect recipient antigen presentation by donor APC (Shlomchik *et al.*, 1999). Chronic GvHD is a multi-organ disease, which can be graded according to its severity. In its severe form it carries a high risk of death due to infectious complications, bone marrow and multi-organ failure.

The risk of GvHD is influenced by the type of transplant and genetic differences between patient and donor as described earlier. It is also greatly influenced by the conditioning regimen received by patients. Traditionally conditioning was myeloablative (destructing the recipients bone marrow activity). Regimens included high dose total body irradiation (TBI) or fractionated (less toxic) TBI in combination with cyclophosphamide (an alkylating agent, whose metabolite forms DNA crosslinks leading to cell death), cyclophosphamide combined with busulfan (alkylating agent) or BEAM (consisting of the carmustine 1,3-bis (2-chloroethyl)-1-nitroso-urea (BCNU, alkylating agent), etoposide (inhibitor of the enzyme topoisomerase II that is involved in DNA replication, its cytotoxic effect results from inhibiting or altering DNA synthesis), cytarabine (a synthetic pyrimidine nucleoside, whose metabolite can inhibit DNA polymerase via competition with deoxycytidine triphosphate, incorporation into DNA and RNA may also contribute to the cytotoxic effect) and melphalan (alkylating agent)). Since the 1990s HSCT was also performed with non-myeloablative regimens (reviewed in (Mackinnon *et al.*, 2004)). This reduced intensity conditioning (RIC) is less toxic and primarily immunosuppressive. HSCT performed with RIC enables the treatment of patients, including those with myeloma (Peggs *et al.*, 2003a), myeloid leukaemia and myelodysplastic syndrome (Tauro *et al.*, 2005), Hodgkin's Disease (Thomson *et al.*, 2008) and Non-Hodgkin's lymphoma (Peggs *et al.*, 2005b), some of whom were too

unfit to endure the toxicity of myeloablative conditioning and therefore could not be treated with HSCT previously. They use lower doses of chemo-radiotherapy within conditioning regimens aimed at achieving a mixed chimaerism in the patients' haematopoietic cells and rely on eradication of tumours by the graft *versus* tumour effect of donor T cells from the graft or additional donor lymphocyte infusions (DLI) (Bloor *et al.*, 2008, Peggs *et al.*, 2005a, Peggs *et al.*, 2007) (reviewed in (Peggs and Mackinnon, 2001b, Peggs and Mackinnon, 2001a)). Conditioning included the immunosuppressive agent fludarabine (adenine analogue) given before low dose TBI or different combinations of fludarabine, cyclophosphamide and melphalan (Einsele *et al.*, 2003, Peggs *et al.*, 2004, Perez-Simon *et al.*, 2003). The choice of conditioning in recent HSCT depends on the type of underlying disease as well as the age and co-morbidity and therefore tolerance to toxicity by the patients. (reviewed in (Copelan, 2006) and (Hart and Peggs, 2007))

GvHD prophylaxis can be achieved by administration of immunosuppressive drugs. Most commonly used are methotrexate (MTX), which inhibits cellular proliferation; cyclosporine A (CSA), which suppresses antigen induced transcription of the IL2 gene and corticosteroids, such as prednisolone, which can reduce several immune responses involved in GvHD pathogenesis.

More specifically, MTX is a folic acid antagonist that inhibits the enzyme amidophosphoribosyltransferase that is involved in purine ribonucleotide synthesis. Its action as a decoy substrate inhibits critical biochemical reactions, thereby exhibiting a cytostatic effect on proliferating cells but also inducing renal, hepatic and gastrointestinal toxicity (Fairbanks *et al.*, 1999).

CSA is a cyclic peptide composed of 11 amino acids, which can be extracted from fungi. It binds to cyclophilin and thereby inhibits calcium-induced activation of the enzyme calcineurin. Calcineurin is a key enzyme involved in the signal transduction pathway resulting in IL2 expression for the activation of several immune cells such as T cells (Wiederrecht *et al.*, 1993).

Prednisolone and its pro-drug prednisone are steroid hormones, more specifically glucocorticoids, which are involved in a wide range of physiologic systems. Steroid hormones are characterised by their three hexane and one pentane ring. The principal naturally occurring glucocorticoid is cortisol, which is derived from cholesterol, synthesised and secreted by cells in the adrenal gland and regulated by negative feedback mediated via the hypothalamic-pituitary-adrenal (HPA) axis by

occupancy of its receptor. Although activity via membrane receptors is possible (Bartholome *et al.*, 2004), the glucocorticoids mostly act via cytosolic receptors. The hormone can passively diffuse across cell membranes, where it activates its main receptor, the glucocorticoid receptor (GR), which upon dimerisation translocates to the nucleus where it can bind to specific glucocorticoid response elements (GRE) and regulate transcription of a number of genes. GR is expressed at different levels in all cell types. The synthetic glucocorticoid prednisolone is four times more potent in its anti-inflammatory effect than cortisol. This anti-inflammatory effect is achieved by blocking synthesis of proinflammatory cytokines and their effects such as the Janus kinases - Signal Transducers and Activators of Transcription (Jak-STAT) signalling pathway, inhibition of proliferation or apoptosis of immune cells, inhibition of antigen processing and reduction of adhesion molecules as well as repressing transcription factors such as the nuclear factor-kappa B (NF-κB), which explains their effect on genes without GRE in their promoters (reviewed in (Cole, 2006) and (Webster *et al.*, 2002)) However, the broad range of effects mediated by glucocorticoids also results in a variety of adverse effects including cutaneous side effects, wound healing disorders, growth failure, osteoporosis, adverse effects on the eyes such as cataract or glaucoma, diabetes, cardiovascular effects such as hypertension, weight gain and neuropsychatic symptoms (Schacke *et al.*, 2002).

In addition to MTX, CSA and corticoids, GvHD prophylaxis or treatment can also be achieved by administration of alternative immunosuppressive drugs. These supplementary pharmaceuticals include tacrolimus (FK506), which is structurally different but acts in a similar way as CSA (Peters *et al.*, 1993); sicrolimus (rapamycin), which is a structural analogue of FK506 binding to the same immunophilin but acting at a later stage in T cell cycle progression by blocking cytokine-mediated signal transduction pathways (Sehgal, 1995); and mycophenolate mofetil (MMF). The latter inhibits *de novo* synthesis of guanosine monophosphate (Allison *et al.*, 1993). This, in turn leads to decreased levels of guanosine triphosphate and hence deoxyguanosine triphosphate (Allison and Eugui, 2000). This results in a cytostatic effect on cells, which is most pronounced in T cells as they require *de novo* synthesis of purine nucleotides (Allison and Eugui, 2005). Other treatments tested for steroid refractory GvHD include daclizumab (a humanised monoclonal antibody to the IL2 receptor) (Perales *et al.*, 2007), extracorporeal photopheresis (EPE) (Setterblad *et al.*, 2008), infliximab (anti-TNFα monoclonal antibody) (Sleight *et al.*, 2007), thalidomide (Kulkarni *et al.*, 2003) and clofazimine (Rzepecki *et al.*, 2007).

All of the pharmaceuticals mentioned above are associated with severe adverse effects (such as effecting the thymus) and morbidity. The most effective means of reducing GvHD development is T cell depletion (TCD) of the graft but TCD may also lead to increased graft failure, Epstein-Barr virus associated lymphoproliferative disorders and disease recurrence after bone marrow transplantation (relapse) due to the elimination of the graft *versus* tumour response. TCD can be achieved by *ex vivo* or *in vivo* administration of a humanised monoclonal antibody directed against cluster of differentiation 52 (CD52), also called Alemtuzumab or Campath-1H (Kottaridis *et al.*, 2000, Kottaridis *et al.*, 2001) or antithymocyte globulin (ATG) (Kroger *et al.*, 2005). An indirect form of TCD is graft selection for CD34 expressing progenitor cells.

Immunosuppression resulting from the conditioning or post transplant immunosuppressive treatment employed for the prevention or treatment of GvHD give rise to a high risk of infectious complications after HSCT. Bacterial sepsis can occur early in the course of transplantation whereas viral infections such as those caused by CMV usually occur after engraftment. Fungal infections such as those caused by aspergillus may occur after the onset of neutropenia until engraftment. Early recognition and treatment of these infections are vital. Following engraftment, the ongoing risk of infection relates to the degree of immunosuppression.

1-3 The role of CMV in HSCT

Latent CMV infection is frequently reactivated post HSCT. In 1999 the risk of CMV antigenaemia (which is a measure of CMV replication, see section 1-5) at 1 year post HSCT from sibling donors was reported to be between 61 % and 69 % with antiviral prophylaxis using aciclovir or foscarnet (compare section 1-4) respectively (Bacigalupo *et al.*, 1999). In comparison, 40 - 45 % (depending on whether grafts were T cell depleted or not) of recipients of autologous HSCT experience episodes of CMV infections as measured by DNAemia (Peggs *et al.*, 2003d). The most serious of the manifestations of CMV is pneumonia, which is more frequent post allogeneic than autologous HSCT (Enright *et al.*, 1993). Despite treatment 6.3 % of allogeneic HSCT patients still developed CMV pneumonia at a mean of 188 days after transplantation and with an associated mortality rate of 76 % (Nguyen *et al.*, 1999). Overall, similar percentages (24 % and 25 %) of CMV seropositive (and thereby high-risk) patients undergoing either reduced-intensity or myeloablative HSCT may still develop CMV disease by one year post transplantation despite preventative measures (Junghanss *et al.*, 2002). Therefore potential HSCT donors and recipients are typed for their CMV status.

In cases where only a CMV seropositive donor can be found for a CMV seronegative patient, the exclusive use of blood products that have been leukocyte depleted diminishes the risk of primary infection.

Inflammation induced by the conditioning prior to HSCT (described above) can activate transcription factors such as NFκB, which stimulate the CMV MIEP and allow viral reactivation from latency (Fietze *et al.*, 1994).

Immunological impairment due to conditioning for HSCT upsets the balance between viral immune escape strategies and effective immune responses in the transplant recipient (compare sections 1-6 and 1-9). During the first months post allogeneic HSCT two thirds of patients fail to develop a protective $CD8^+$ T cell response against CMV. They are at risk of CMV reactivation. Dissemination of CMV in the blood and subsequent infection of multiple organs contribute to CMV pathogenesis and can cause fatal pneumonia, myelosuppression, gastroenteritis, retinitis, myelitis and occasionally graft failure (Sissons and Carmichael, 2002). A large number of autograft transplant recipients restore CMV specific T cell responses during the first 3 months and are therefore susceptible to infection for a shorter time period after transplantation than allograft recipients (Reusser *et al.*, 1997).

CMV pathology occurs through a number of direct and indirect effects. It has been implicated in allograft rejection secondary to up-regulation of adhesion molecule expression and cytokine secretion in response to CMV infection. Up-regulation of the intercellular adhesion molecule 1 (ICAM-1) and the lymphocyte function-associated antigen 3 (LFA-3) by CMV has been demonstrated and is accentuated rather than abrogated by the antivirals ganciclovir and foscarnet (Craigen and Grundy, 1996). Up-regulated secretion of proinflammatory cytokines such as IL6 by CMV infection was also demonstrated (Carlquist *et al.*, 1999). This can result in further up-regulation of adhesion molecules and MHC antigens, thereby enhancing the recognition of host tissue by donor T cells.

During prolonged immunosuppression, lytic CMV infection can lead to loss of tissue function resulting in interstitial pneumonitis, hepatitis or gastrointestinal disease (Peggs and Mackinnon, 2004b). This pathogeneicity can be further enhanced by viral induced inflammation. CMV disease can also result in retinitis (Crippa *et al.*, 2001) and rarely encephalitis (Stroup *et al.*, 2007).

Given the proinflammatory properties of CMV described earlier, it is not surprising that an association between pre-transplant CMV seropositivity and an

increased risk of GvHD was demonstrated (Bostrom *et al.*, 1990). A study by Broers and colleagues showed that CMV seropositivity of either HSCT recipient or donor proved an independent risk factor for developing acute GvHD suggesting that CMV seropositivity affects transplantation outcome by increasing the incidence of acute GvHD (Broers *et al.*, 2000). CMV has been shown to prolong and increase graft inflammation (Lautenschlager *et al.*, 1997). CMV infection-induced cytokine production in recipient endothelium is likely to worsen the cytokine storm observed during aGvHD (described earlier). Endothelial cells are known to produce inflammatory cytokines such as IL6 after infection with CMV, which can increase leukocyte adhesion (Grefte *et al.*, 1993). Consistent with that IL6, amongst other cytokines such as IL1 and TNFα, was shown to be important during the initial phase of T cell activation before T cells are specifically activated by recipient alloantigens (Lichtman A, 1997). The association between pre-transplant CMV seropositivity and an increased risk of GvHD may also be mediated by viral peptides cross-reacting with minor antigens. Nachbaur and colleagues suggested that donor-derived HLA-A*0201 restricted CMV specific $CD8^+$ T cells can cross-react with HLA-A*0201 restricted recipient minor histocompatibility antigen (Nachbaur *et al.*, 2001). In accordance with that Cwynarski and colleagues (Cwynarski *et al.*, 2001) reported detection of CMV specific $CD8^+$ T cells in only one of seven CMV seronegative recipients receiving allogeneic HSCT from seropositive donors, with this recipient being the only one of the seven to experience aGvHD. Gandhi and colleagues later suggested, that CMV specific $CD8^+$ T cells derived from a seropositive donor might persist in a seronegative recipient with severe GvHD due to alloreactivity (Gandhi *et al.*, 2003a). CMV DNAemia in such a recipient was undetectable indicating absence of primary CMV infection in the study by Gandhi and colleagues. They had determined the origin of cells by analysis of DNA sex chromosome and single nucleotide polymorphism and demonstrated donor origin of all virus specific $CD8^+$ T cell clones. Alloreactivity of CMV specific $CD8^+$ T cells was also suspected in one of the patients of the study described here (section 3-2.1.3). A direct correlation of CMV specific immune responses with the development of GvHD has been demonstrated for aminopeptidase N, also called CD13 (compare description of viral entry receptors in section 1-1). CMV particles contain host derived CD13 molecules and therefore CMV infection of HSCT patients can result in the development of autoantibodies against host CD13, which correlates with the development of chronic GvHD (Soderberg *et al.*, 1996).

An influence in the opposite direction (that is the influence of CMV pathogeneicity by GvHD complications) is also apparent. The slow regeneration of the immune system after stem cell transplantation is often protracted by the presence of GvHD and its immunosuppressive prophylaxis and treatment. As a result recipients of T cell depleted stem cell grafts are at higher risk of reactivating CMV (Couriel *et al.*, 1996). Accordingly investigations of CMV specific $CD8^+$ T cell responses post HSCT must consider GvHD complications and any immunosuppressive regimen including TCD in patients. The source of transplant is another important factor contributing to CMV immune regeneration with more rapid reconstitution after transplantation of peripheral blood stem cells (PBMC) mobilised with granulocyte colony stimulating factor (G-CSF) from allogeneic donors than reconstitution after conventional bone marrow transplantation (BMT) (Korbling *et al.*, 1995). The development of non-myeloablative conditioning that enables wider application of HSCT has increased the rate of opportunistic infections. The increased use of immunosuppressive treatment such as Campath-1H in this setting is associated with prolonged T cell depletion (Morris *et al.*, 2003) and a high incidence of CMV infection (Chakrabarti *et al.*, 2002). Nevertheless Campath-1H was shown to not adversely affect overall transplant-related mortality (Peggs, 2004c). High-risk (CMV seropositive) patients undergoing HSCT with reduced intensity conditioning develop CMV complications as late as 1 year post transplantation, which is much later than complications observed post myeloablative conditioning (Junghanss *et al.*, 2002). The study described here demonstrates CMV replication at even later time periods in patients who received reduced-intensity conditioning with Melphalan and Fludarabine in combination with T cell depletion by Campath-1H *in vivo* and who could be studied for longer than 500 days post HSCT (patients 3, 11, 21 and 30, refer to Figure 3-1/Figure 3-16, Figure 3-7, Figure 3-15 and Figure 3-22 respectively).

In summary CMV is thought to play a complex role in haematopoietic stem cell transplantation with a dual mechanism of pathogenesis. On the one hand the virus replicates and is cytolytic in infected tissue, which leads to loss of tissue function. On the other hand pathogeneicity in organs is indirectly enhanced by the inflammatory effect of CMV and its inhibition of engraftment of transplanted cells resulting in maintained immunodeficiency. This is supported by findings that bone marrow cells including $CD34^+$ cells can be infected with CMV (Maciejewski *et al.*, 1992) and that CMV is myelosuppressive (Sing and Ruscetti, 1990).

1-4 Pharmacological interventions for the prevention and treatment of CMV in HSCT recipients

Pharmacologic intervention is highly efficient but since these drugs inhibit viral DNA replication or translation of viral ribonucleic acid (RNA) during primary or reactivated CMV infections, they have no impact on the latent phase of the viral life cycle and therefore cannot eradicate the infection. Long-term therapy is limited by drug resistance mutations and substantial toxicity of the drugs, which can lead to viral recurrence.

Drugs currently licensed for the treatment of CMV infection include ganciclovir, its oral pro-drug valganciclovir, foscarnet, cidofovir and fomivirsen whereas (val) aciclovir is licensed for prophylaxis. Figure 1-4 depicts the molecular structures of these antivirals and their resemblance of components involved in DNA elongation. Fomivirsen's mechanism of action is not involved in DNA replication. Therefore its structure is not shown in this figure but within the text later.

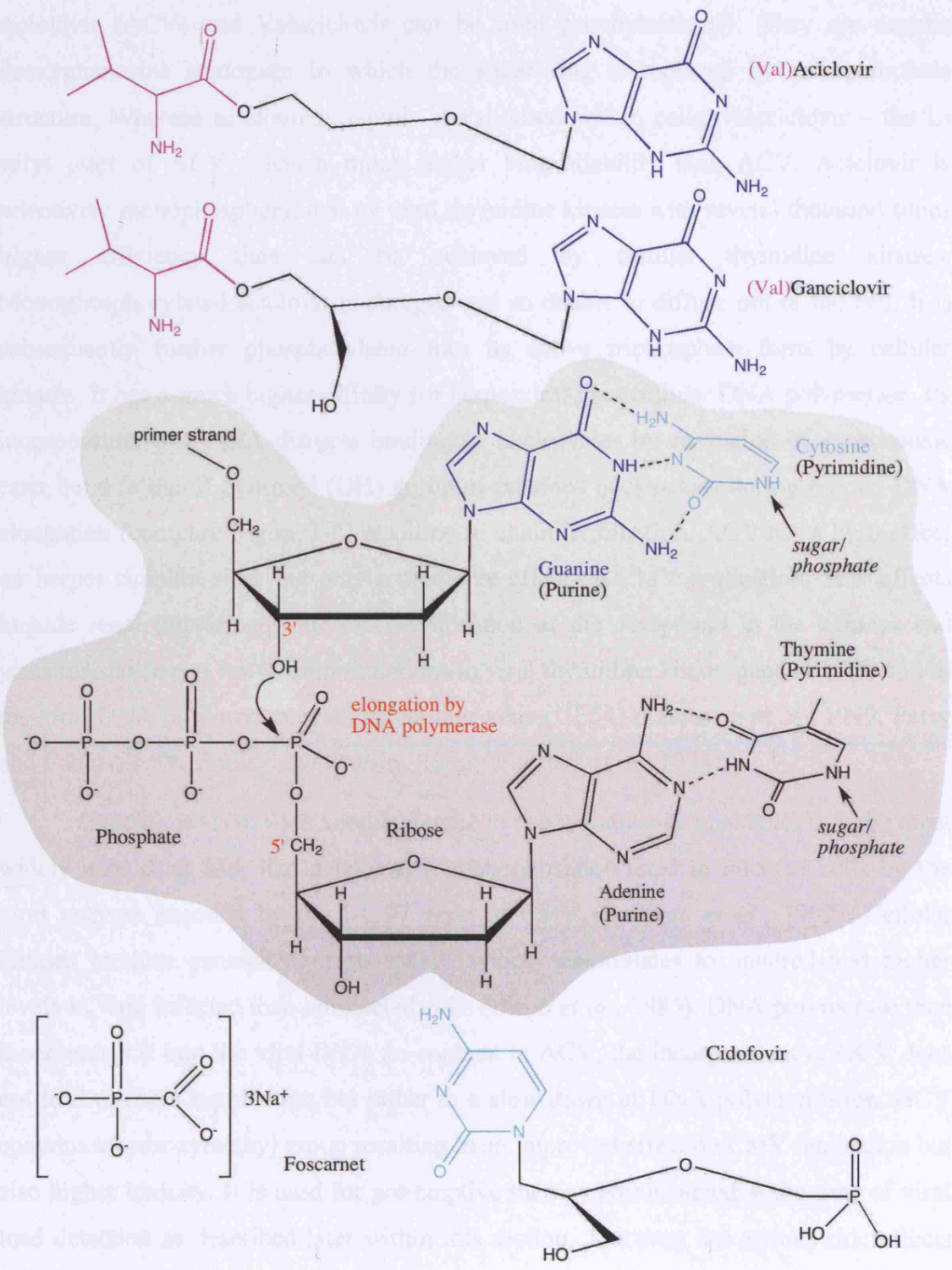


Figure 1-4 Antiviral pharmaceuticals

This figure illustrates DNA elongation in the grey shaded area and molecular structures of common pharmaceuticals used to inhibit CMV replication above and below. (Val) ganciclovir and aciclovir are guanosine analogues whereas foscarnet is a pyrophosphate analogue and cidofovir is a cytosine analogue. The incorporation of these compounds into the growing chain of viral DNA during the process of 3' to 5' addition of nucleotides to the sugar phosphate backbone of DNA by the DNA polymerase results in inhibition of DNA replication. The figure was generated using ChemDraw Ultra Version 11.0.1 (CambridgeSoft).

Aciclovir (ACV) and Valaciclovir can be used prophylactically. They are acyclic deoxyguanosine analogues in which the sugar ring is replaced by an open-chain structure. Whereas aciclovir is rapidly metabolised within cells, valaciclovir – the L-valyl ester of ACV - has a much higher bioavailability than ACV. Aciclovir is selectively monophosphorylated by viral thymidine kinases with several thousand times higher efficiency than can be achieved by cellular thymidine kinases. Monophosphorylated aciclovir is charged and so unable to diffuse out of the cell. It is subsequently further phosphorylated into its active triphosphate form by cellular kinases. It has a much higher affinity for herpesviral than cellular DNA polymerase. Its incorporation into DNA disrupts binding of nucleosides by formation of a phosphate ester bond to the 3' hydroxyl (OH) group of previous nucleosides during normal DNA elongation (compare Figure 1-4) resulting in chain termination. ACV has a high effect on herpes simplex virus but only a moderate effect on CMV replication. Side effects include renal impairment due to crystallisation of the compound in the kidneys and virus resistance can result from mutations in viral thymidine kinase gene (UL97) and/or the viral DNA polymerase catalytic subunit gene (UL54) (Lowance *et al.*, 1999, Perry and Faulds, 1996, Smiley and Murray, 1996, Wagstaff *et al.*, 1994).

Ganciclovir (GCV) is another synthetic deoxyguanosine analogue. It is the most widely used drug and, like aciclovir, is monophosphorylated in infected cells by the viral enzyme encoded by the UL 97 gene of CMV (Sullivan *et al.*, 1992). Cellular kinases produce ganciclovir-triphosphate, which accumulates to hundred-fold higher levels in viral infected than uninfected cells (Biron *et al.*, 1985). DNA polymerase then incorporates it into the viral DNA. In contrast to ACV, the incorporation of GCV does not lead to chain termination but rather to a slow down of DNA polymerisation. GCV contains a hydroxymethyl group resulting in an improved effect on CMV replication but also higher toxicity. It is used for pre-emptive therapy administered at the time of viral load detection as described later within this section. The drug has serious side effects including inhibition of growth of haematopoietic stem cells and neutropenia. Therefore G-CSF is used in combination with GCV to increase neutrophil numbers (Crumpacker, 1996). The treatment with GCV has also been demonstrated to limit CMV specific immune reconstitution after HSCT (Li *et al.*, 1994). Extended ganciclovir therapy can lead to drug resistance mutations. These occur most frequently in the UL97 gene inhibiting anabolism of the drug or, to a lesser extent, in the DNA polymerase gene (UL54) inhibiting the incorporation of ganciclovir-triphosphate into the viral DNA (Smith *et al.*, 1997). Valganciclovir is the valyl ester pro-drug of GCV and is

hydrolysed to ganciclovir by intestinal and hepatic esterases. It acts by the same mechanism as GCV but has an increased oral bioavailability (Jung and Dorr, 1999).

Foscarnet (FCN, trisodium phosphonoformat) is an alternative treatment option to GCV. It is a pyrophosphate analogue acting as inhibitor of viral DNA polymerase by directly inhibiting its pyrophosphate binding site. It blocks the cleavage of the pyrophosphate group of the nucleoside triphosphate added to the growing DNA chain at concentrations that do not affect human DNA polymerases. This drug does not require cellular activation (Wagstaff and Bryson, 1994). Responses to FCN can result in resistance mutations in UL54. However, the compound is active against CMV variants expressing mutated UL97 proteins found in GCV resistant clinical isolates and can therefore be used in combination with GCV to reduce drug resistance (Sissons and Carmichael, 2002). Main adverse effects of FCN include high nephrotoxicity, electrolyte imbalances and poor bioavailability requiring intravenous administration (Nyberg *et al.*, 1989).

Cidofovir (CDV) is a cytosine analogue. It does not require a viral function for its activation. It is a phosphonate, structurally equivalent to nucleoside monophosphate but without the charge which would prevent the molecule crossing the plasma membrane. It only needs two phosphorylations by cellular enzymes to be converted to its active form. The diphosphorylated forms then act as a chain terminator of viral DNA synthesis and two consecutive incorporations efficiently terminate DNA elongation. They have a higher affinity for the viral than cellular DNA polymerases. CDV is active against CMV variants with UL97 mutations but UL54 mutations can result in resistance. It has a prolonged half-life in comparison to ganciclovir and foscarnet, which enables less frequent administration (Reusser, 2000) but possesses low bioavailability that necessitates intravenous administration. Its severe renal toxicity necessitates administration with probenecid to prevent kidney damage (Lalezari, 1997) and renders it a second-line therapy for the treatment of CMV retinitis (De Clercq and Holy, 2005).

Fomivirsen is an oligodeoxynucleotide of the sequence 5'-GCGTTTGCTCTTCTTCTTGCG-3' complementary to the major immediate early 2 (IE2) mRNA of CMV blocking translation of IE2 encoding mRNA (Azad *et al.*, 1993). Due to modified nucleosides and/or sugars it has a long half-life. Although not directly involved in viral genome replication, the crucial role of IE protein production in initiating the replication of CMV as described in section 1-1 make them an important target for antiviral intervention. Fomivirsen can be administered by intraocular injection

and is highly efficient but due to adverse affects its use is restricted to the treatment of CMV retinitis (Biron, 2006) and so does not effect systemic CMV.

The role of intravenous immunoglobulin therapy is controversial. Some studies suggested a benefit for patients treated with immunoglobulin in combination with ganciclovir post HSCT (Reed *et al.*, 1988), whereas others reported a limited value of this combined treatment for the outcome of established cytomegalovirus pneumonia after HSCT (Verdonck *et al.*, 1989). A more recent randomised trial has shown no benefit from immunoglobulins used prophylactically (Ruutu *et al.*, 1997), but successful treatment of established CMV disease was reported in individual patients (Chow *et al.*, 1992, Hiyoshi *et al.*, 1997, Koriyama *et al.*, 2004, Weng *et al.*, 2003).

Initial prophylactic interventions, usually with GCV, using continuous treatment during the first 100 days post HSCT led to prolonged neutropenia resulting in increased risk of bacterial and fungal infections and therefore no improved overall survival in patients. Subsequently, pre-emptive strategies were developed based on detection of CMV reactivation (compare section 1-5) prior to the development of disease and administration of antiviral treatment such as GCV until CMV becomes undetectable (Boeckh *et al.*, 1996). The advantage of pre-emptive treatment over antiviral prophylaxis is that the former targets only those patients who are at maximum risk for developing CMV diseases and minimises exposure of others. Pre-emptive treatment reduces the incidence of CMV disease during the first 100 days post transplantation(Goodrich *et al.*, 1991). However, patients receiving antiviral treatment during this time as well as patients with high viral load and patients developing chronic GvHD are at increased risk for late-onset CMV disease, which is now the main CMV-related complication after allogeneic HSCT (Boeckh *et al.*, 2003, Einsele *et al.*, 2000, Zaia *et al.*, 1997). Antiviral treatment early after HSCT is likely to block the development of protective immunity. Continued prophylaxis with GCV in combination with CMV viral load monitoring is not feasible because the association of GCV with neutropenia and opportunistic infections may offset its beneficial effects (Winston *et al.*, 1993). This may contribute to the risk of uncontrolled late-onset CMV reactivation in patients who are not actively monitored any more. Additional CMV preventative approaches are needed in the HSCT setting and non-pharmacological treatment alternatives such as adoptive CMV specific $CD8^+$ T cell immunotherapy might prove beneficial.

1-5 Monitoring CMV replication post HSCT

CMV disease (with the exception of retinitis) cannot be diagnosed based on clinical manifestations alone. Viral isolation from patient samples is diagnostic for CMV infection but diagnosis of CMV disease traditionally requires histopathology, cytology or culture based methods to provide evidence for presence of the virus in affected tissues (de la Hoz *et al.*, 2002) These methods are time consuming and place patients at risk of delayed treatment. Increasing CMV load in the blood is thought to precede clinical disease in the majority of HSCT patients (Gor *et al.*, 1998). Thus, detection methods based on various viral parameters were developed in addition to traditional methods to identify patients at risk for CMV disease.

Viral parameters currently used for monitoring CMV replication are viraemia (isolation of infectious virus from peripheral blood leucocytes), antigenaemia (quantitative measurement of pp65 in peripheral blood leucocytes using immunofluorescence), DNAemia (quantitative measurement of CMV DNA or copy number in peripheral blood leucocytes, plasma or whole blood by either polymerase chain reaction (PCR) or hybridisation technique) and RNAemia (measurement of IE or L mRNA in peripheral blood leucocytes or whole blood by reverse transcriptase PCR or nucleic acid sequence based amplification).

Early tissue culture based methods were time consuming, poorly reproducible and of low sensitivity. In contrast, newer methods based on quantitative antigen detection or molecular amplification that are rapid, highly sensitive and reproducible are now available (reviewed in (Boeckh and Boivin, 1998)) and used routinely to monitor HSCT patients (Peggs, 2004a).

However, it should be noted that the inability to detect systemic CMV DNAemia or antigenaemia during routine monitoring does not preclude subclinical CMV reactivation at localised tissue sites in HSCT patients (discussed by (Gandhi *et al.*, 2003a)).

1-6 Induction of CMV specific immune responses

A variety of natural immune defence mechanisms against viral intruders such as CMV exist in the human host. The epithelium is the first line of defence. Epithelial cells are joined by tight junctions providing a physical barrier against most microorganisms. CMV circumvents this first line of defence by infection through bodily fluids such as urine, saliva, blood, tears, semen or breast milk.

The first components of the immune system that will subsequently be encountered by the pathogen are the cells and molecules of the innate immune system. Innate recognition occurs by preformed receptors that recognise structures on a broad range of pathogens. This inherited arm of the immune system thereby enables an immediate response. Receptors can either recognise pathogens and their products directly or recognise host molecules that are upregulated by cells under conditions of stress. Viral double stranded RNA, which is generated during the life cycle of CMV and unmethylated cytosines in "cytosine and guanine separated by a phosphate" (CpG) dinucleotides can be recognised directly by Toll-like receptors (TLR) and a role for TLR 3 and 9 was demonstrated for the innate immune defence against mouse cytomegalovirus infection (Tabeta *et al.*, 2004). Indirect means of recognition include those via NKG2D ligands, of which several are known to exist (Eagle *et al.*, 2006). Although CMV produces UL16 protein that can retain NKG2D ligands in the inside of infected cells as an immune evasion strategy, only some (MICB, ULBP1, and ULBP2 but not MICA and ULBP3) of the NKG2D ligands are affected by this strategy (Dunn *et al.*, 2003, Welte *et al.*, 2003). NKG2D ligands can activate receptor-bearing cells such as NK cells and trigger multiple signalling cascades (Sutherland *et al.*, 2002). The importance of NK cells is demonstrated in patients with NK cell deficiencies, who are very susceptible to CMV infection and disease (Biron *et al.*, 1989). NK cells can recognise virus-infected cells by their failure to express self major HLA class I molecules as a result of the viral infection (missing-self hypothesis) (Ljunggren and Karre, 1990). On ligation with activating receptors mentioned above they are able to exert immediate action against infected cells. Innate immune responses can lead to direct killing of some infected cells, early production of inflammatory cytokines with antiviral activity and production of chemokines that recruit inflammatory cells into the infected tissue. The complement cascade plays an important role in this response. It involves C proteins that can bind to the Fc region of antigen-antibody complexes, thereby mediating antibody-dependent cytotoxicity. C proteins can also directly bind to

viral proteins. Subsequent activation of a cascade of enzymatic events leads to deposition of complement complexes on the surface of virus-infected cells resulting in lysis. Furthermore complement fragments produced during the process of enzymatic cleavage induce soluble immune mediators and can opsonise virus, thereby initiating or amplifying immunological defence mechanisms that might normally only occur at low level (Hirsch, 1982). CMV has evolved strategies to escape immune control mediated by the complement cascade. The presence of C control proteins in virus particles reduces lysis mediated by deposition of complement complexes (Spear *et al.*, 1995), and virus-encoded Fc receptor homologues may entice antibodies away (Keller *et al.*, 1976). Interferon α and β that are produced during viral infection (Goodbourn *et al.*, 2000) are also important mediators of innate effector functions and were found to stimulate cross-presentation (Le Bon *et al.*, 2003), which may play an important role for adaptive immune defence against CMV as will be described later within this section. Apoptotic infected cells and debris are delivered to regional lymph nodes by tissue derived DC or other APC priming the adaptive immune response mediated by lymphocytes (Steinman *et al.*, 1999).

In contrast to innate immune responses, immune cells of the adaptive arm of the immune response (lymphocytes) utilise an antigen receptor repertoire that is a result of developmental processes that vary between individuals (compare section 1-8.1). The variability of lymphocyte receptors increases the possibility of detecting any antigen that may be encountered throughout life. Lymphocytes originate from haematopoietic lymphoid progenitors in the bone marrow and can be distinguished into two main cell types. T lymphocytes (T cells) mature in the thymus and develop membrane bound T cell receptors whereas B lymphocytes (B cells) mature in the bone marrow and develop B cell receptors both in transmembrane and soluble form (the soluble form is alternatively called immunoglobulin or antibody). Upon first encounter of antigen, lymphocytes must first proliferate and differentiate before being effective against pathogens. The adaptive arm of the immune response is therefore slower to respond than the innate arm of the immune response. However, unlike the innate response, an adaptive response leaves behind a pool of memory lymphocytes and antibodies that can act immediately upon a subsequent encounter of the same pathogen. B-lymphocyte derived CMV immunoglobulin (Ig) might be beneficial when administered to transplant patients (compare section 1-4). However its beneficial effect is believed to be directed against indirect inflammatory effects of CMV (Kazatchkine and Kaveri, 2001) and to be tolerance inducing rather than directly antiviral (Hoetzenecker *et al.*, 2007). Maternal

humoral immunity before conception is important for the prevention of damaging congenital CMV infection in the newborn (Fowler *et al.*, 1992). In contrast, humoral immunity against CMV may not play an important role post HSCT (Munoz *et al.*, 2001). In addition, individuals suffering from common variable immunodeficiency who lack the ability to produce sufficient antibodies in response to exposure to viral and other pathogens (Bayry *et al.*, 2005) were demonstrated to have CMV specific T cell responses and no symptoms of CMV disease. This indicates that CMV replication can be controlled by T cells in the absence of B cells (Kopycinski, 2006).

T cells have been shown to be the main mediators of the immune defence against CMV and since their presence inversely correlates with infection and disease, adoptive therapy has been considered as potential therapy in immunocompromised patients (Greenberg *et al.*, 1991, Quinnan *et al.*, 1984, Quinnan *et al.*, 1982, Reusser *et al.*, 1991, Riddell *et al.*, 1991b, Walter *et al.*, 1995). T cells are sub-classified according to the expression of either CD4 or CD8 co-receptors. Both $CD4^+$ and $CD8^+$ T cells are required for complete regeneration of CMV immunity (Foster *et al.*, 2002) and a hierarchy of immune control functions of $CD8^+$ T cells, NK cells and $CD4^+$ T cells has been suggested in mice (Polic *et al.*, 1998). $CD4^+$ T cells are thought to play a critical role in determining the fate of $CD8^+$ T cells and it was demonstrated that in the absence of the former, $CD8^+$ T cell mediated control of persistent herpesvirus is lost (Cardin *et al.*, 1996). The probability for patients infected with human immunodeficiency virus (HIV) to develop CMV disease has been reported to be 13 %, 3 % and 0 % in the presence of $CD4^+$ T cell counts of less than 50 cells/µl, 50-100 cells/µl and 100 cells/µl respectively (Gerard *et al.*, 1997). Spiegel and colleagues described the presence of CMV specific $CD8^+$ T cells without effector function in the absence of low levels of $CD4^+$ T cells in this patient group (Spiegel *et al.*, 2000). In addition to $CD4^+$ T cells with helper function, cytotoxic subsets of $CD4^+$ T cells targeting CMV have recently been observed with properties and function similar to those of CMV specific $CD8^+$ T cells (Crompton *et al.*, 2008).

There are several lines of evidence that $CD8^+$ T cells may play one of the most important roles in protective immunity against CMV. Delayed reconstitution of $CD8^+$ T cells correlates with CMV disease in allogeneic bone marrow transplant patients (Li *et al.*, 1994) and a study by Reusser and colleagues demonstrated the association of CMV specific $CD8^+$ T cells with protection from CMV disease in autologous transplant patients (Reusser *et al.*, 1997).

Since $CD8^+$ T cells play such an important role in protective immunity against CMV, this thesis focuses on investigating the fine specificity and of these cells and their correlation with protection against CMV in HSCT patients. $CD8^+$ T cells exert their specificity to antigen through their T cell receptors. These molecules were demonstrated to bind antigen in the form of peptides presented by HLA molecules (compare section 1-2) at the surface of antigen presenting cells (Zinkernagel and Doherty, 1974). The process by which CMV specific antigens are presented to and induce $CD8^+$ T cells is reviewed below.

After viral infection of cells, CMV encoded proteins are digested into peptide fragments by the proteasome and other intracellular proteases (Coux *et al.*, 1996), which are thought to generate the final carboxy-terminal residues of HLA binding peptides. The resulting peptides are translocated into the lumen of the endoplasmatic reticulum (ER) by the transporter associated with antigen presentation (TAP) (Lankat-Buttgereit and Tampe, 2002), which spans the membrane of the ER. In the ER, peptides usually undergo additional trimming at the amino terminus that is mainly facilitated by ER associated aminopeptidase (ERAAP) (Serwold *et al.*, 2002) before associating with the heavy chains of HLA class I molecules. Noncovalent interaction results from sequence-dependent interaction between side chains of the peptide (anchors) and pockets of the peptide-binding groove of the HLA class I molecule on the one hand and a conserved hydrogen bond network formed by non-polymorphic amino acids at each end of the peptide-binding groove of the HLA and the peptide on the other hand. Thereby peptide-binding specificity is determined by the first mentioned interaction whereas the last mentioned interaction restricts the lengths of peptides to generally eight to ten residues, although larger peptides can bind by bulging in the middle. A series of chaperone proteins facilitate the partial folding of class I HLA and its association with $ß_2$ microglobulin ($ß_2m$) in the ER before it can interact with TAP. These chaperones include the TAP binding protein tapasin, the thiol oxidoreductase ERp57, calnexin and calreticulin, which all assist protein folding by promoting the formation of disulfide bonds, regulating the redox state during initial folding of proteins or retaining unfolded proteins in the ER. It also includes the immunoglobulin-binding protein (BiP) although its role is yet undetermined. Once the peptides are transported to the ER and are trimmed by ERAAP, peptide editing occurs. This involves peptide loading and exchange to achieve binding of high affinity peptides to the awaiting HLA, a process that is mediated by protein disulfide isomerase (PDI). The stabilised HLA/$ß_2m$/peptide

complex can then be transported to the plasma membrane via the Golgi network. (reviewed by (Antoniou and Powis, 2008, Cresswell *et al.*, 2005, Jensen, 2007))

The ER is an interconnected membrane network held together by the cytoskeleton. It is continuous with the outer layers of the nuclear envelope and proteins can be shuffled between the ER and the Golgi apparatus. The complexity of the ER is unaccounted for in Figure 1-5, which schematically illustrates the process of antigen processing for presentation of peptide fragments on HLA class I molecules on the surface of cells.

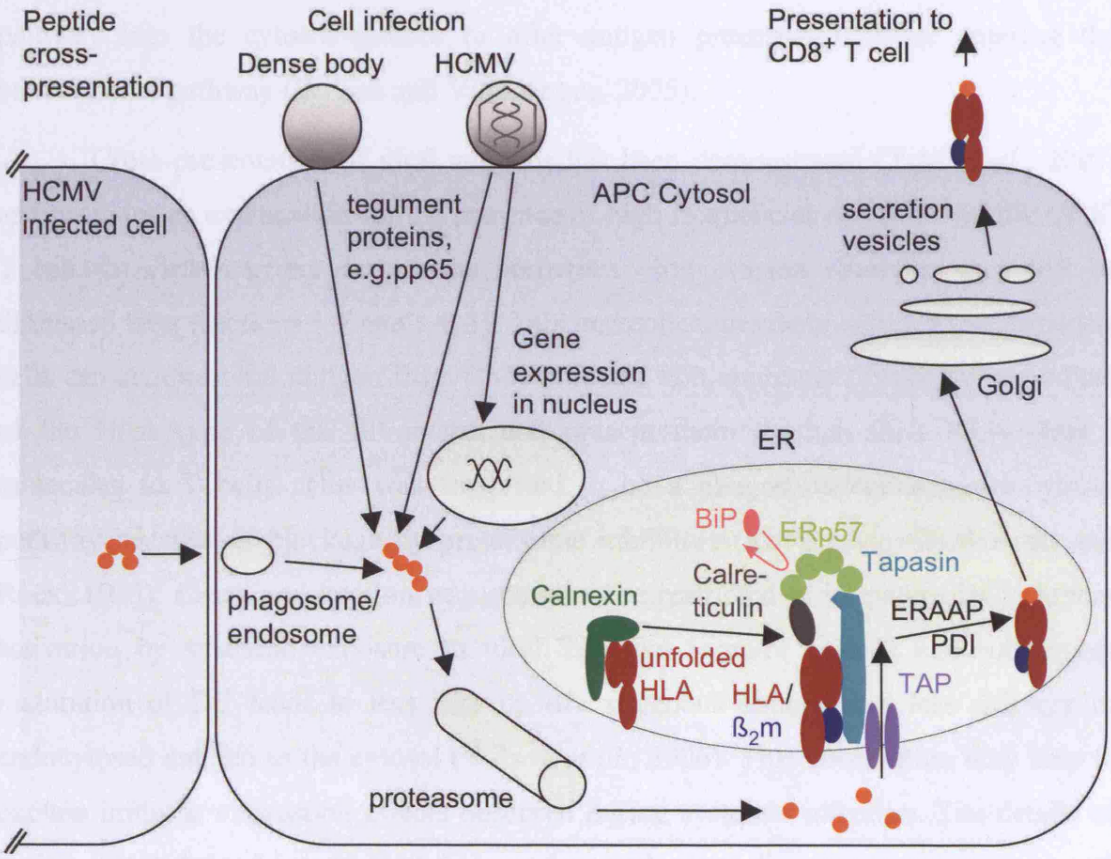


Figure 1-5 Processing of CMV antigen for presentation by HLA class I

The processing of CMV peptides by APC is illustrated. The HLA class I heavy chain initially assembles with the chaperon calnexin that initiates folding. Subsequently $ß_2$m and HLA associate with the peptide loading complex involving calreticulin, TAP, tapasin and ERp57. BiP has been described to bind transiently but its role remains undetermined so far. Peptides generated by the proteasome and other intracellular proteinases are translocated into the ER. This is usually achieved via TAP. Peptide editing results in peptides of the appropriate sequence to bind to HLA. The stabilised HLA/$ß_2$m/peptide complex then dissociates from the peptide-loading complex and is transported to the cell surface. Cross-presented peptides may bind by alternative TAP dependent or TAP independent mechanisms. The graph was created based in information from (Antoniou and Powis, 2008, Cresswell *et al.*, 2005, Jensen, 2007, Pepperl *et al.*, 2000, Tabi *et al.*, 2001, Topilko and Michelson, 1994).

In contrast to the endogenous pathway of antigen processing described above, exogenous proteins that a cell has endocytosed are usually processed by a different pathway leading to presentation by HLA class II molecules that can be recognised by $CD4^+$ T cells. This is not further discussed here because this project focuses on $CD8^+$ T cells recognising peptides presented via HLA class I molecules. However, CMV derived peptides taken up exogenously (cross-priming) or from subviral dense bodies (alternative loading) can also associate with HLA class I to induce a $CD8^+$ T cell response and both of these pathways are illustrated along with the conventional pathway in Figure 1-5. Endocytosed exogenous antigens are believed to exit the endosomal pathway into the cytosol (before or after antigen processing) before entering the conventional pathway (Wilson and Villadangos, 2005).

Cross-presentation of viral antigens has been demonstrated (Tabi *et al.*, 2001) and provides an explanation for the presence of high frequencies of CMV specific $CD8^+$ T cells in virus carriers despite the numerous viral evasion strategies that will be discussed later (sections 1-7 and 1-9.3). Tabi and colleagues demonstrated that dendritic cells can acquire viral antigen from CMV infected non-apoptotic fibroblasts regardless of the HLA type of the fibroblasts and present them through their HLA class I molecules to T cells. This was suggested to be a phagosome/endosome-to-cytosol pathway because of blockage by proteasome inhibitors (Kovacsovics-Bankowski and Rock, 1995). Cross presentation was shown to be restricted to immature DC whereas activation by systemic exposure to viral Toll-like receptor ligands and subsequent maturation of DC leads to less take up of exogenous antigen and less delivery of endocytosed antigen to the cytosol (Wilson *et al.*, 2006). This observation may help to explain immune exhaustion effects observed during systemic infection. The details of various TAP dependent and TAP independent pathways of cross-presentation are still being discovered. Delivery of peptides from virally infected cells to APC (compare Figure 1-5) may involve phagocytosis (Ackerman *et al.*, 2003) or a direct gap-junction-mediated transfer (Neijssen *et al.*, 2005). Importantly, since cross-presentation results in presentation of viral antigen by non-infected cells, production of CMV encoded gene products with immunomodulatory function (as described in sections 1-7 and 1-9.3) is avoided.

Dense bodies (DB) are enveloped spherical structures that lack viral capsid and DNA. In addition to properly assembled new virions (compare Figure 1-1 on page 29), infected cells were shown to also release these non-infectious defective particles (Craighead *et al.*, 1972) including DB and non-infectious enveloped particles (NIEP) that resemble virions but lack DNA. They consist mainly of glycoprotein and tegument protein with pp65 being its major component (Baldick and Shenk, 1996, Fiala *et al.*, 1976). DB and NIEP were shown to be able to enter cells with the same kinetics (<60 seconds after contact with cell membrane) and mode of penetration as complete virus particles enabling delivery of their protein components into the cell (Topilko and Michelson, 1994). Consistent with that the ability of DBs to induce significant $CD8^+$ T cell responses in the absence of infectious virus was recently demonstrated in mice (Pepperl *et al.*, 2000).

Activation of naïve $CD8^+$ T cells involves stimulation through the T cell receptor, which recognises CMV derived peptides bound to MHC class I molecules on infected cells and requires costimulation by antigen presenting cells (compare Figure 1-11 on page 71). Upon encounter of antigen, lymphocytes stop migrating and enlarge. Activated CMV specific $CD8^+$ T cells increase in size, thereby becoming lymphoblasts. Lymphoblast transformation was induced by short-term stimulation of T cells with phytohaemagglutinin (PHA) to induce mRNA levels throughout this project and is therefore described briefly. The generation of lymphoblasts results from an energetic event, during which metabolism increases and the production of new RNA and proteins is induced. Therefore ribosomes, which are necessary to synthesize proteins, associate with the ER resulting in a rough appearance of the latter, the cytoplasm increases and the plasma membrane increases its number of microvillus and its phagocytic activity. This process results in enlargement of the entire cell. An illustration of this transformation induced by PHA is shown in Figure 1-6.

Figure 1-6 T cell lymphoblast formation

Surface morphology (left: unstimulated, right: PHA stimulated, both 8,000x) is shown, adapted from Hoffmann and colleagues (Hoffmann *et al.*, 1977).

Lymphoblasts undergo clonal proliferation and become effector $CD8^+$ T cells that can produce cytokines such as IFNγ or directly kill CMV infected cells via FAS or perforin dependent mechanisms (compare sections 1-9.1 and 1-9.2). In contrast to their naïve counterparts, antigen-experienced $CD8^+$ T cells can recognise their target cells with little or no requirement of co-stimulation, thus enabling killing of virally infected cells other than APC (compare section 1-9). At this stage cells have changed their expression of adhesion molecules, which enables them to migrate between endothelial cells at the sites of infection (Masopust *et al.*, 2001). Effector $CD8^+$ T cells are attracted to these sites by cytokines released by local inflammatory cells. After viral clearance, most virus-specific $CD8^+$ T cells die by apoptosis but some survive as a population of memory cells which can become cytotoxic and proliferate upon restimulation with CMV antigen.

Naïve $CD8^+$ T cells circulate in the lymphatic system between blood and secondary lymphoid organs via specialised post capillary vessels called high endothelial venules (HEV) that are only expressed on lymphoid tissue. Their migration involves interaction between L-selectins (CD62L), chemokine receptors (CCR7) and integrins (LFA1) with their ligand counterparts expressed on HEV. These are the peripheral lymph node addressin (PNAd), CCL21 and ICAM1 respectively (Masopust *et al.*,

2001). In contrast, memory T cells can be classified into central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) according to their expression of lymph node homing molecules and immediate effector function. Whereas T_{CM} ($CD62L^+CCR7^+$) are thought to reside preferentially in secondary lymphoid organs, T_{EM} ($CD62L^-CCR7^-$) and short-lived effector T cells can migrate directly into non-lymphoid tissue to exhibit immediate effector function (Lewis *et al.*, 2007, Mora and von Andrian, 2006). This was shown to involve tissue specific receptor interaction (Picker *et al.*, 1993) that may be conferred by activation with tissue specific APC or by the local environment, in which a naïve T cell becomes activated. As an example, interaction of T_{EM} with the endothelium of small intestinal blood vessels was shown to involve the integrin $\alpha_4\beta_7$ (Berlin *et al.*, 1993), chemokine receptor CCR9 (Zabel *et al.*, 1999) and a higher affinity LFA-1 with the ligands mucosal addressin cell adhesion molecule (MAdCAM) (Nakache *et al.*, 1989), CCL25 (Hosoe *et al.*, 2004) and ICAM-1 respectively. Evidence of naïve $CD8^+$ T cells accessing non-lymphoid environment in some circumstances, however, is growing and awaits further studies on the potential functionalities of these cells (Lewis *et al.*, 2007).

1-7 CMV strategies to limit the priming of an immune defence

Probably to facilitate persistence in the host, and thereby enhancing the chance for transmission to a new host, human CMV devised several strategies for avoiding immune detection. One of these is the limitation of gene expression to the minimum during latency. A second strategy involves the replication in tissues that have less stringent immune surveillance. Epithelial cells of the salivary gland do not express sufficient HLA class I molecules to mediate virus clearance by $CD8^+$ T cells and thereby allow virus shedding into body fluids for viral transmission between individuals (Hengel *et al.*, 1998). As a third strategy, the virus does express several factors designed to silence host defence mechanisms. Some examples of those are mentioned later in this section and other factors directly influencing $CD8^+$ T cell effector functions are mentioned in section 1-9.3.

CMV prevents virions from being exposed to the host immune defence by reducing apoptosis through the action of IE1 and IE2 genes (Zhu *et al.*, 1995). In addition to that CMV encodes gene products with immunomodulatory function that can thwart recognition by host effector cells. These include some US proteins, which decrease cell surface expression of MHC class I molecules in CMV infected fibroblasts *in vitro* (Jones *et al.*, 1995) and are therefore thought to interfere with cytotoxic $CD8^+$ T cell immune surveillance. The product of US3 is expressed rapidly but transiently and

prevents egress of MHC class I proteins from the ER to the Golgi apparatus (Lee *et al.*, 2000). In addition, US10 can delay the trafficking of MHC class I molecules through the ER to the cell surface (Furman *et al.*, 2002). The products of the genes US2 and US11 are expressed in the E phase and act in a similar way to redirect MHC class I from the ER into the cytosol leading to their ubiquitin-dependent proteasome-mediated degradation (Gewurz *et al.*, 2001, Shamu *et al.*, 2001). Both products appear to use different dislocation complexes (reviewed in (Antoniou and Powis, 2008)). US2 and US3 were also shown to result in downregulation of HLA class II (Hegde and Johnson, 2003, Hegde *et al.*, 2002). The product of US6 is expressed in the L phase and prevents peptide loading by binding to TAP and thereby retaining it in the lumen of the ER (Hewitt *et al.*, 2001). CMV carries an abundance of genes involved in immune modulation of the host other than those interfering with peptide presentation, which will be described in section 1-9.3.

Interference of CMV with peptide presentation would be most efficient if the virus would selectively down-regulate viral antigen presenting alleles, while at the same time preserving other alleles to act as inhibitors of NK cell activation. A varying efficiency of immune evasion proteins depending on the HLA allele targeted has been observed and may explain the predominance of $CD8^+$ T cells targeting peptides presented by resistant HLA alleles rather than non-resistant alleles. Several HLA-B alleles including HLA-B*0702 were found to be resistant to effects of the US2 protein whereas others such as HLA-A*0201 are not (Barel *et al.*, 2003). Consistent with that Lacey and colleagues demonstrated a relative dominance of HLA-B*0702 restricted $CD8^+$ T cells to CMV pp65 in individuals sharing HLA-A*0201 and HLA-B*0702 alleles (Lacey *et al.*, 2003), which may hold true for other responses as well.

The strategies used by CMV to evade immune responses of the host is likely to play a role in shaping the host T cell response and may partly explain the immunodominance of $CD8^+$ T cells directed to antigens like pp65 that can be presented by infected cells before expression of the US genes (compare Figure 1-5 on page 54). Although the sequential expression of US proteins targets the efficiency of cell mediated immunity of the host, $CD8^+$ T cells remain critical in the control of CMV replication and it has been shown that infected cells remain targets throughout the replicative cycle *in vitro* despite the expression of viral genes that downregulate class I MHC expression (Riddell and Greenberg, 1997). Specific cytokines can limit the immune evasion potential of CMV from $CD8^+$ T cell control (compare section 1-9.3)

showing that there exists a highly balanced relationship between host and pathogen (Hengel *et al.*, 1995). Manley and colleagues demonstrated that the immune evasion proteins do not prevent the development of a diverse CMV specific $CD8^+$ T cell response *in vivo*. Interferon gamma (IFNγ) production of $CD8^+$ T cells from healthy CMV seropositive donors was greater towards fibroblasts infected with US gene deletion mutant RV798 than towards the wild type AD169 CMV strain (Manley *et al.*, 2004). It is unknown whether $CD8^+$ T cells specific for CMV antigens that are not presented by permissively infected fibroblasts *in vitro* are activated and maintained at relatively high frequency *in vivo* but several explanations seem possible. It may be possible that the US proteins do not impair antigen presentation in fibroblasts or non-fibroblasts *in vivo* as efficiently as they do in fibroblasts *in vitro*. This may be due to different kinetics of the US expression in different cell types leading to incomplete antigen presentation impairment (Manley *et al.*, 2004). It was shown that deletion of genes expressing products with immunomodulatory function greatly affects antigen presentation *in vitro* but has little effect on the magnitude of the overall murine anti CMV $CD8^+$ T cell response *in vivo* (Gold *et al.*, 2002). Sylwester and colleagues recently demonstrated immunogenicity of 33 different open reading frames, which were recognised by $CD8^+$ T cells in several CMV carriers each (Sylwester *et al.*, 2005). These findings suggest that part of the $CD8^+$ T cell response is induced by cross presentation (compare section 1-6) because genes expressing products with immunomodulatory function should act only in cells that express them and therefore should effect only direct priming of T cells by APC that present endogenous antigen. Indeed induction of $CD8^+$ T cells targeting structural and non-structural CMV proteins by crosspriming was demonstrated (Tabi *et al.*, 2001).

1-8 $CD8^+$ T cell specificity

Specific $CD8^+$ T cell recognition of virus-infected cells is accomplished by the interaction of T cell receptors (TCR) on the effector cells with HLA/viral peptide complexes on infected cells. All human nucleated cells express up to six different major class I HLA molecules (compare section 1-2) and up to 250,000 of each of the HLA class I molecules were estimated to present peptides on the surface of a cell (Parham and Ohta, 1996).

TCRs are highly variable antigen-recognition structures. Conventional $CD8^+$ T cells express approximately 10^4 identical TCRs on their surface (reviewed in (Mazza and Malissen, 2007)). They are disulfide-linked, membrane-bound heterodimers consisting of α and β chains for most mature T cells (γδ T cells are not considered in this chapter). Each chain is the result of rearrangement of multiple copies of variable (V), joining (J) and, in the case of the ß-chain, diversity (D) segments (compare Figure 1-9 on page 67) during T cell maturation and subsequent joining to one of the constant (C) regions. The α and ß chains of mature T cell receptors then contain an amino-terminal variable (V) region and a constant (C) region, followed by a short hinge region (where α and ß chain are linked by a disulfide bond), a transmembrane region and the cytoplasmic tail.

1-8.1 T cell receptor rearrangement during T cell development

The TCRα chain is encoded on chromosome 14. Its V region is encoded by two DNA segments, the V gene segment and the J gene segment, with the latter located closely to the C region. During developmental gene rearrangement of T cells in the thymus, the V and J gene segments are joined. This enzymatic process is guided by conserved, noncoding DNA sequences called recombination signal sequences (RSS). The newly formed exon is joined to the C region sequence by RNA splicing after transcription. The TCRß chain V region is encoded in three gene segments on chromosome 7 (compare Figure 1-9). In addition to the V and J gene segments, there is a third gene segment, the D gene segment, lying between them. During rearrangement, D and J segments are joined first before joining with V. During the process of gene rearrangement, the sequences between rearranging genes are deleted in the form of circular excision products called TCR excision circles (TREC). Multiple copies of all gene segments exist. The combination of randomly selected gene segments of each type results in great diversity of the TCR V regions. Figure 1-7 illustrates the TCR Vß gene rearrangement

process that is responsible for generating variable T cell receptors. Additionally a structural model of the TCR protein, in which the V-D-J junction is located in the centre of the TCR - HLA/peptide interaction region is illustrated in Figure 1-8 on page 65.

This figure, adapted from (van Dongen *et al.*, 2003) illustrates the sequential TCR Vß gene rearrangement steps (here Vß4-Dß2-Jß2.3), transcription, RNA splicing and translation that finally result in the surface expression of a T cell receptor on a T cell. The formation of extrachromosomal TRECs during this recombination process is also indicated.

Figure 1-7 T cell receptor rearrangement

The T cell development described above occurs from common lymphoid progenitors expressing CD34 that have migrated from the bone marrow to the thymus. There, cytokine signalling and interaction with cells present in the thymus guides the different stages of thymocyte differentiation. TCRβ rearrangement occurs first followed by rearrangement of TCRα. Once a productive ß chain gene rearrangement has occurred, it pairs with an invariant pre-α chain. Subsequent expression of a pre-TCR triggers phosphorylation and degradation of RAG (product of the recombination activating gene, the main enzyme complex involved in the rearrangement of TCR genes). This stops further ß chain rearrangement and ensures allelic exclusion. Cell proliferation and expression of co receptor are induced. Only after proliferation has ceased, RAG-1 and RAG-2 are transcribed again and the TCRα chain can be rearranged. It then pairs with the ß chain to produce the final T cell receptor. (refer to (Kuklina, 2006) for a review)

Resulting thymocytes express low levels of TCRα/ß receptors and undergo selection depending on their recognition of self-MHC/peptide complexes. TCR recognition of MHC/peptide ligands results in thymocytes receiving signals for survival and further differentiation into either $CD4^+$ or $CD8^+$ single positive thymocytes (positive selection). Subsequently high avidity interactions elicit signals that result in deletion of thymocytes. This process of negative selection contributes to the clonal deletion of self-reactive cells thereby avoiding autoimmunity. Only about 3 - 5 % of developing thymocytes with TCR recognising MHC/peptide ligands with low avidity are thought to survive the selection in the thymus (Egerton *et al.*, 1990, Goldrath and Bevan, 1999). This selection process enriches cells that are potentially reactive to foreign antigen, but not to self-antigen, presented by self-MHC. (reviewed in (Huseby *et al.*, 2008))

In addition to the diversity created by the joining of different gene segments, further diversity of the TCR between gene segments of the V-D-J (TCRβ) or V-J (TCRα) junction results from random incorporation and deletion of nucleotides during the enzymatic process of gene rearrangement (N- and P-nucleotides). This alters the junctional regions in lengths (compare section 1-8.2), thereby creating regions not encoded in the germline DNA, and results in the high variability of V regions necessary for the recognition of many potentially encountered antigens. The potential diversity created for TCR heterodimers has been calculated to be approximately 10^{15} (Davis and Bjorkman, 1988). The TCR repertoire following thymic selection is between 10^7 and 10^8 (Arstila *et al.*, 1999).

The variability of the resulting TCR V region is not distributed evenly. When α and ß chains are paired in the TCR molecule hypervariable sequences form loops in the resulting protein structure leading to three complementarity-determining regions (CDR) of the antigen-binding site amongst less variable framework regions. CDR1 and CDR2 are thought to interact primarily with the HLA and are encoded in the germline V segment genes, whereas CDR3 encompasses the V-J, or in case of the ß-chain, the V-D-J junction (Wilson *et al.*, 1988), is located in the centre of the TCR-HLA/peptide interaction region and is thought to interact primarily with the peptide ligand. A structural model for the interaction between TCR and MHC/peptide (determined by crystallography) is shown in Figure 1-8.

Figure 1-8 Model of the structure of an MHC class I/peptide-TCR complex

This figure shows a backbone tube representation of the interaction of a T cell receptor (TCR) with a major histocompatibility complex (MHC) class I/peptide. The MHC, consisting of its three α domains associated with ß$_2$-microglobulin (ß$_2$m), is on the bottom with the peptide shown as large tube in yellow. The TCR is above with the CDR α1 and α2 coloured pink, CDR ß1 and ß2 coloured blue and the CDR3 coloured yellow (adapted from (Garcia *et al.*, 1996)).

The CDR3 of the ß-chain is the most diverse part of the TCR and TCR diversity can therefore be experimentally estimated by analysing the length of this region. The method for distinguishing Vß transcripts according to CDR3 length was initially described by Cochet *et al.* (Cochet *et al.*, 1992). It has been called spectratyping by Gorski *et al.* (Gorski *et al.*, 1994) and immunoscope by Pannetier *et al.* (Pannetier *et al.*, 1995) and is briefly explained below.

1-8.2 Estimation of T cell receptor diversity

Junctional diversity generated during somatic recombination as described above results in CDR3 regions differing in size by multiples of 3 nucleotides whereas rearrangements that would be out-of-frame are rarely detected in PBMC (Gorski *et al.*, 1994). Vß CDR3 size variation is limited. Seven to 12 amino acid residues account for more than 80 % of all functional rearrangements (Liu *et al.*, 1995a). Vß spectratyping involves the generation of complementary DNA (cDNA) followed by amplification of a segment of the T cell receptor ß chain that spans the CDR3 region with a fluorescently labelled primer specific for the Cß region and primers specific for Vß. As it is not possible to design a single primer that can anneal to all Vß segments of the 24 families that exist, multiple PCR reactions with different primers need to be performed. Figure 1-9 illustrates the location of functional TCR Vß genes.

Centromeric

Figure 1-9 TCR Vß locus at 7q34

This figures illustrates the location of TCR Vß (TRBV) genes designated according to the nomenclature by Rowen and colleagues (Rowen *et al.*, 1996). Of the total number of 91-94 TRB genes that exist per haploid gene, 82-85 (64-67 TRBV, 2 TRBD, 14 TRBJ and 2 TRBC) are localised on chromosome 7 at 7q34 (shown) and 9 orphans are localised on chromosome 9 (not shown). Of these 82-85 TRB genes, 56-65 genes are functional (40-48 TRBV, 2 TRBD, 12-13 TRBJ and 2 TRBC) and TRBV genes can be allocated to 21-23 subgroups depending on the nomenclature used. Adapted from (Lefranc, 2001b) (http://imgt.cines.fr).

A multiplex PCR approach using 7 reactions each with a mix of differentially labelled Vß family specific primers and one Cß primer was used in the project described here (compare Table 2-7 on page 165). The PCR products of different length can be resolved by denaturing gel electrophoresis. The ratio of transcripts per cell has been demonstrated to be fairly constant. Therefore the amount of PCR product as measured by fluorescence intensity in each size peak indicates the number of clonotypes and therefore TCR diversity. The deletion and addition of nucleotides during the rearrangement of T cell receptors is random. Therefore the CDR3 lengths in cord blood approximate to a Gaussian distribution (Garderet *et al.*, 1998). Clonal expansions such as those of CMV specific $CD8^+$ T cells (Weekes *et al.*, 1999a, Weekes *et al.*, 1999b, Weekes *et al.*, 1999c) result in changes in the relative peak heights. Antigenic stimulation results in skewing of the Gaussian distribution in CDR3 lengths of

peripheral T cells from adults with increasing age. Predominance of only a few size classes in a spectratype indicates oligoclonality.

The thymic selection shapes the preimmune TCR repertoire from which antigen can drive the selective expansion of T cell clones with preferred TCR that subsequently dominate the T cell response to an antigen (McHeyzer-Williams *et al.*, 1999). The TCR repertoire used for the recognition of some peptide-MHC complexes may vary widely between individuals reflecting a private TCR usage. Some viral infections, however, are known to induce presentation of peptide-MHC complexes, which generate T cell subsets expressing similar CDR3 ß loops in different individuals. This public TCR usage can for example be observed in HLA-B8 expressing individuals after Epstein-Barr virus (EBV) infection by generation of a prominent Epstein-Barr nuclear antigen 3 (EBNA-3) specific T cell responses with a dominant TCR (Argaet *et al.*, 1994). Another example for public TCR usage can be found for HLA-A2 restricted T cell responses towards influenza virus (Moss *et al.*, 1991). It has recently been suggested that structurally dominant *versus* absent peptide features may determine the selection of a diverse *versus* limited TCR repertoire of $CD8^+$ T cell responses to influenza A virus infection in mice respectively (Turner *et al.*, 2005). In contrast, peptides bound to HLA in a bulged form, which are likely to be rare, may only be accommodated by a few TCRs. $CD8^+$ T cell responses with such extremely restricted TCR sequences have been reported for bulged 11mer and 13mer target structures (Tynan *et al.*, 2005, Miles *et al.*, 2005). Alternatively, prevalence of virus specific T cell clones with public TCR usage may be due to a functional advantage conferred by the CDR3 motifs. A competitive advantage due to superior $CD8^+$ T cell avidity was demonstrated for influenza A specific $CD8^+$ T cells in HLA-A2 expressing individuals, in whom a dominant public TCR Vß17 amino acid sequence was up to 10,000-fold more avid than subdominant clones that were expanded *in vitro* after removal of $V\beta17^+$ cells (Lawson *et al.*, 2001). Subdominant clones were also shown to occur *in vivo* during HLA-B8 restricted EBV specific $CD8^+$ T cell responses (Argaet *et al.*, 1994). The selective expansion of $CD8^+$ T cells expressing high affinity TCRs may allow for more rapid destruction of virus-infected cells whereas the persistence of subdominant $CD8^+$ T cell clones with heterogeneous (low affinity) TCRs may provide a mechanism to cope with pathogen mutation.

TCR usage of CMV specific T cells has been investigated previously demonstrating public TCRs with expansions in Vß1 and other Vß families in the context of HLA-A*0201 when using spectratyping (Peggs *et al.*, 2003c). The knowledge of TCRs used in the T cell response to different CMV antigens may be useful for enhancing the detection during the monitoring of CMV responses in high-risk immunodeficient patients or for the development of treatment modalities using TCR gene transfer. Besides their role in protection from CMV dissemination and disease, $CD8^+$ T cell responses to CMV can have an adverse effect on the immune fitness of infected individuals by contributing towards shaping the overall $CD8^+$ T cell repertoire. Ouyang and colleagues demonstrated that a large fraction of the entire $CD8^+$ T cell population in individuals more than 85 years old consists of T cells specific for a single CMV epitope. The fraction of these cells secreting IFNγ upon antigen stimulation is lower than in younger individuals and may additionally result in increased susceptibility to infectious disease by limiting the T cell repertoire available for responses to other antigens in the elderly (Ouyang *et al.*, 2003). Therefore the diversity of TCRs used by CMV specific $CD8^+$ T cells has important implications for patients beyond their protection from CMV.

1-8.3 T cell receptor excision circles (TRECs)

TCR gene recombination as described in section 1-8.1 results in formation of T cell receptors, whereby TRECs are formed as by-products (compare Figure 1-7, page 62). TRECs can be used experimentally to estimate the number of naïve T cells. This is based on the fact that these extrachromosomal DNA fragments are stable within cells (Livak and Schatz, 1996). TREC content is therefore high in naïve T cells that have recently rearranged their TCR genes and did exit the thymus. Since TRECs are not duplicated during mitosis, they are diluted with every cell division (Takeshita *et al.*, 1989) and therefore TREC content is low in activated proliferating T cells.

In αβ T cells, TRECs are produced during the rearrangement of both, TCRα encoding and TCRβ encoding, genes. Many potential gene recombinations bring about different potential TRECs. However, 67 % of all αβ T cells produce similar signal and coding joint TRECs during the deletion of the TCRD locus when the TCRα genes are rearranged (Douek *et al.*, 1998). This is illustrated in Figure 1-10. Accordingly these TRECs can be used to estimate the number of naïve T cells amongst peripheral cells.

Figure 1-10 Signal joint and coding joint TREC

This figure illustrates the TCRD locus embedded within the TCRA locus and formation of the signal joint (sj) TREC by ligation of recombination signal sequences flanking the δ-rec locus and ψ-Jα locus on the top. The coding joint (cj) then becomes part of a second TREC when recombination of TCRAV to TCRAJ occurs (illustrated on the bottom). Adapted from (Douek *et al.*, 1998).

Reconstitution of T cells post HSCT depends on the expansion of memory T cells and the generation of new T cells from progenitors (Bomberger *et al.*, 1998). The measurement of TRECs amongst peripheral cells is useful to determine if reconstituted cells stem are either newly generated (high TREC content) or did expand from mature T cells (low TREC content). In comparison, determination of thymopoietic capacity on the basis of $CD45RA^+$ phenotyping of naïve T cells is of limited value as these cells may very rapidly convert into memory T cells of $CD45RO^+$ phenotype (Picker *et al.*, 1993) and memory T cells can also convert back into $CD45RA^+$ expressing T cells (compare section 1-9).

1-9 $CD8^+$ T cell differentiation, effector function & CMV immune evasion

T cells that completed their development in the thymus including rearrangement of their TCR (refer to section 1-8.1 for details) circulate between blood and peripheral lymphoid organs. These cells that have not yet encountered their specific antigen are termed naïve T cells. The engagement of the TCR of a $CD8^+$ T cell recognising a CMV derived peptide bound to HLA class I molecule on the surface of an antigen presenting cell in combination with costimulatory signals results in activation (compare section 1-6) and subsequent clonal proliferation with the development of daughter cells bearing the same TCR. Activation and subsequent signalling is complex. A simplified illustration is provided in Figure 1-11. It involves molecular clustering that leads to formation of an

immunological synapse and interaction of an ever-growing list of signalling molecules (reviewed in (Razzaq *et al.*, 2004)).

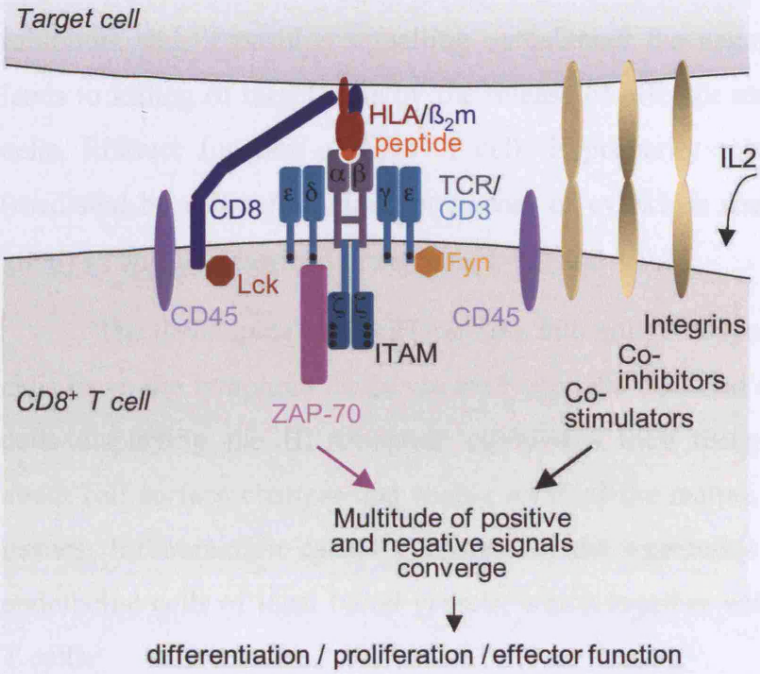


Figure 1-11 CD8$^+$ T cell activation

This figure illustrates how triggering of the TCR/CD3 complex that bound to specific HLA/peptide on target cells is combined with various other signals received via costimulators, coinhibitors and integrins. An early effect of TCR engagement is the activation of members of the Src tyrosine kinase family (CD3 associated Fyn and CD8 associated Lck) via dephosphorylation by the phosphotyrosine phosphatase CD45. Activated leukocyte-specific protein tyrosine kinase (Lck) and Fyn are instrumental for phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domains of CD3 chains. This, in turn, creates binding sites for further proteins with Src-homology type 2 domains (SH2). One of these is the tyrosine kinase Zap-70, which becomes then activated by phosphorylation by one of the Src-like kinases, which, together with Zap-70, phosphorylate several downstream substrates and initiate further signalling cascades. Activation of transcriptions factors for several genes (with one of the most important being IL2 and IL2 receptor) in combination with complex modifications including mobilisation of calcium, release of retained preformed receptors and granules and internalisation of surface receptor finally results in differentiation, proliferation and effector functions of T cells. TCR signalling can be tuned by the affinity for its ligand. High affinity peptide binding results in phosphorylation of all ITAMs of receptors of the CD3 complex that can lead to full T cell activation. In contrast, low affinity peptide binding can result in incomplete phosphorylations and thereby may for example induce cytolysis but not proliferation. TCR signals only result in T cell activation of naive CD8$^+$ T cells when combined with costimulatory signals (for example CD28, NKG2D, CD40L and 4-1BB with the latter being expressed on activated T cells). These signals are further tuned by inhibitory signals (for example KIR, CTLA-4 and Fas, being expressed or sensitive only after T cell activation), integrins and cytokines. Naïve CD8$^+$ T cells do not express IL2 and its receptor. These are induced upon TCR engagement and costimulation, resulting in stimulation of growth, differentiation and survival of antigen-selected CD8$^+$ T cells. IL2 production triggered by TCR and costimulation, however, may not be sufficient for full activation and CD4$^+$ T cells can provide additional cytokines. This figure was created based on information mainly from (Andersen *et al.*, 2006, Medema and Borst, 1999, Vivier and Malissen, 2005).

Recognition of target cells by $CD8^+$ T cells results in oligomerisation of TCR molecules that triggers a complex cascade of downstream intracellular signalling events. Signals originating from TCR triggering can be tuned by co-stimulators, integrins and co-inhibitors and, if positive signalling outbalances the negative signalling, it eventually leads to killing of target cells by the release of effector molecules by effector $CD8^+$ T cells. Effector function of $CD8^+$ T cells is primarily achieved by direct cytotoxicity (mediated by cell-cell contact) or release of cytokines such as IFNγ (Andersen *et al.*, 2006) as will be described in sections 1-9.1 and 1-9.2.

The developing cells differentiate into antigen-experienced $CD8^+$ T cells. These cells leave the lymphoid tissue via the lymphatic duct and enter the blood to find target cells displaying the HLA/peptide complexes they recognise. Differentiation brings about cell surface changes that enable some of the mature $CD8^+$ T cells to enter other tissues. Inflammation causes a change in the expression of adhesion molecules on endothelial cells of local blood vessels, which together with chemokines attracts $CD8^+$ T cells.

Differentiation is accompanied by expression of different surface molecules and mediators of cytotoxicity. Discrimination of different T cell subsets according to their differentiation stage has been defined using different markers by different investigating groups (amongst others including CD45 isoforms, CCR7, CD28, CD11b, CD57, CD27, CD7, CD62L, granzyme and perforin content, ability to proliferate and/or secrete cytokines and activation markers such as HLA-DR and CD38), which makes comparisons difficult. However the consensus reached is that CMV specific $CD8^+$ T cells are present in heterogeneous phenotypes *in vivo* (Gillespie *et al.*, 2000).

The expression of distinct isoforms of CD45 enables antigen-experienced T cells to respond to TCR mediated signals with little or no co-stimulation. The isomers CD45RA and CD45RO are generated by alternative splicing of mRNA transcripts with the resulting CD45RO proteins exhibiting stronger association with the TCR signalling complex than CD45RA. Both molecules catalyse dephosphorylation of protein tyrosine kinases (Lck, Fyn) triggering subsequent steps of T cell activation (Figure 1-11).

Naïve $CD8^+$ T cells express the costimulatory molecule CD27 and CD45RA (as well as other markers such as the costimulatory molecule CD28 and the lymph node homing receptor CCR7), whereas memory T cells lose the expression of CD45RA. Recently primed virus-specific T cells are mainly of the $CD45RO^+CD27^+$ phenotype with cytolytic activity only after restimulation (Hamann *et al.*, 1997). These $CD27^+$ T

cells irreversibly switch off CD27 expression after stimulation for prolonged periods (Hintzen *et al.*, 1993) followed by additional loss of CD28 expression. Differentiating T cells eventually revert back to expression of $CD45RA^+$ (Hamann *et al.*, 1999, Wills *et al.*, 1999). The readily detectable effector functions of $CD27^-$ T cells, such as high granzyme B expression and direct cytolytic activity (described below), would predict these cells to be critical in the control of virus replication (Bachmann *et al.*, 1999). Accordingly, the majority of CMV specific $CD8^+$ T cells display a $CD45RO^+/CD28^-/CCR7^-$ phenotype with varying expression of CD27 during acute primary infection and most of these cells switch to a $CD45RA^+/CD28^-/CD27^-/CCR7^-$ late differentiation phenotype after recovery (Wills *et al.*, 2002b). Identical T cell clones were found to express either isoform of the CD45 molecules suggesting a common origin of antigen experienced CD45RO and CD45RA expressing CMV specific T cells (Wills *et al.*, 1999). CMV specific $CD8^+$ T cells are enriched for the $CD27^-/CD28^-$ phenotype in HSCT patients (Gandhi *et al.*, 2003a). Furthermore CMV specific $CD8^+$ T cells exposed to antigen for prolonged periods of time are found to express a $CD57^+CD27^-$ or $CD57^+CD28^-$ phenotype that was previously associated with terminally differentiated non-proliferating effector T cells (Kern *et al.*, 1999a, Kern *et al.*, 1996) but was more recently reported as a highly differentiated T cell subset of unique functionality (including the capability of proliferation) late after activation (Chong *et al.*, 2008). Several lines of evidence suggest that highly differentiated CMV specific $CD8^+$ T cells of $CD45RA^+/CD28^-/CD27^-/CCR7^-$ phenotype are actually readily stimulated via alternative costimulatory molecules (Waller *et al.*, 2007) and have the capacity to provide immediate effector function as well as to migrate to draining lymph nodes (reviewed in (Waller *et al.*, 2008)). Following resolution of primary infection, CMV specific $CD8^+$ T cells remain high in immunocompetent individuals, probably to hold the persisting virus in check (Gillespie *et al.*, 2000). They are thought to be periodically stimulated, thus undergoing expansion with each viral reactivation (Weekes *et al.*, 1999a, Weekes *et al.*, 1999b, Weekes *et al.*, 1999c) resulting in memory inflation over time (Kern *et al.*, 1996).

1-9.1 Direct cytotoxicity of $CD8^+$ T cells

The two main mechanisms of induction of direct cytotoxicity by effector $CD8^+$ T cells (and NK cells alike) can be achieved by Fas (CD95) induced apoptosis or by the perforin pathway (Andersen *et al.*, 2006).

The former mechanism involves engagement of the transmembrane protein Fas (CD95) on target cells. This receptor belongs to the tumour necrosis factor (TNF) family. FasL (expressed on activated $CD8^+$ T cells) engagement triggers its homo-trimerisation and subsequent formation of the death inducing signalling complex (DISC) that induces apoptosis (programmed cells death). More specifically, trimerisation involves aggregation of death domains in the cytoplasmic tails of CD95. Subsequently the adaptor protein "Fas-associated protein with death domain" (FADD) binds the death domain of Fas through its own death domain. It also binds to caspase 8 (via a death effector domain) and thereby bridges Fas to caspase 8. Activated caspase 8 then triggers a cascade of caspases (cysteine proteases). It is released from the DISC into the cytosol, where it cleaves other effector caspases, eventually leading to degradation of chromosomal DNA into oligomers of 180 base pairs (bp) and other hallmarks of apoptosis, which is the degradation of cells into apoptotic bodies, which can be recognised and digested by phagocytes without the disruption of target cell membranes and induction of an inflammatory response. (Nagata, 1996)

The perforin pathway is the main mechanism of direct cytotoxicity exerted by effector $CD8^+$ T cells, during which perforin and granzymes (serine proteases) are released into the intercellular space. Both of the mentioned molecules are pre-synthesized and stored in lytic granules that mobilise to the cell surface upon contact with targets to secrete their content directly at the target cells. Perforin then inserts into the target cell membrane forming pores and these are believed to allow cellular uptake of granzymes (especially granzyme B) causing apoptosis of target cells in a caspase-dependent and caspase-independent manner. (reviewed in (Trapani and Smyth, 2002)) Some studies demonstrated that the uptake of granzymes into cells can be perforin-independent (mediated by receptor-mediated endocytosis) and that perforin subsequently mediates entry of granzyme B into the nucleus thereby initiating apoptosis (Shi *et al.*, 1997).

1-9.2 Cytokine secretion of $CD8^+$ T cells

Another antiviral response of effector $CD8^+$ T cells and also innate natural killer (NK) cells encountering CMV infected target cells is the release of the cytokine IFNγ. Binding of this secreted type II interferon results in binding to its cognate transmembrane IFNγ receptor. The intracellular domains of the two chains of the receptor are associated with the janus kinases jak 1 and jak 2 respectively (reviewed in (Bach *et al.*, 1997)). Receptor triggering results in formation of a binding site for the signal transducer and activator of transcription (STAT) 1 and initiation of the Jak-STAT pathway. This pathway involves phosphorylation of STAT1 homodimers, which subsequently translocate to the nucleus and bind to γ activated sequences (GAS) leading to transcription of IFNγ inducible genes (reviewed in (Polic *et al.*, 1998)). Over 200 genes are regulated by IFNγ and these are implicated in antigen presentation, cell adhesion, chemotaxis and inflammation (reviewed in (Boehm *et al.*, 1997)). Main antiviral activity is believed to be mediated by alteration of proteasomes and MHC expression resulting in enhanced antigen processing and presentation. Furthermore INFγ induces transcriptional activation of Fas in target cells resulting in increased Fas-mediated target cell lysis. Adoptive transfer studies have also demonstrated $CD8^+$ T cell function to be dependent on INF γ *in vivo* (Hengel *et al.*, 1994).

IFNγ only moderately inhibits CMV replication *in vitro* (Torigoe *et al.*, 1993). However, its critical role *in vivo* was demonstrated by IFNγ's compensation for viral interference with antigen presentation (compare section 1-6). This was achieved by stimulation of synthesis, assembly and stability of HLA class I molecules resulting in restored antigen presentation in cells exposed to IFNγ before CMV infection thereby limiting the immune evasion potential of CMV from $CD8^+$ T cell control (Hengel *et al.*, 1995).

Effector functions of $CD8^+$ T cells are also mediated by other cytokines (for example TNFα can bind to TNF receptors triggering inflammation and events similar though weaker than to those described for FAS in section 1-9.1).

1-9.3 CMV evasion of immune cell effector functions

Immune evasion allows CMV to increase the available time window for replication thereby facilitating virus shedding. The virus carries an abundance of genes involved in immune modulation of the host, some of which are involved in antigen recognition and were already mentioned earlier (section 1-7).

Important genes encoding products with immunomodulatory functions involving T cell effector functions are briefly described here. Examples of these are genes modulating the expression of MHC (compare section 1-7) and genes expressing MHC class I homologues (UL18 (Chapman *et al.*, 1999)). Additionally genes expressing cytokine homologues (UL111.5A encoding vIL10 (Kotenko *et al.*, 2000)), inducing cytokine expression (UL144 (Atalay *et al.*, 2002)) or those expressing chemokine homologues (UL146 encoding vCXC-1 that mimics human IL8 (Penfold *et al.*, 1999)) and chemokine receptor homologues (US28 (Gao and Murphy, 1994), potentially UL27, UL33 and UL78 (Margulies *et al.*, 1996)) are capable of altering leukocyte behaviour. There is also evidence for viral immune evasion by the induction of TNFβ and subsequent suppression of T cell responses (Michelson *et al.*, 1994). Furthermore CMV encodes genes whose products affect the cell cycle (UL69 (Lu and Shenk, 1999), UL82 encoding pp71 (Kalejta and Shenk, 2003)) and apoptosis (genes encoding 72 kilo Dalton (kDa) IE1 and 80 kDa IE2 proteins (Hagemeier *et al.*, 1992), UL36 encoding vICA (Skaletskaya *et al.*, 2001) and UL37 encoding vMIA (McCormick *et al.*, 2003)). Genes preventing the killing of CMV infected cells by NK cells include UL40, UL18 (both explained below), the HLA class I homologues UL142 (Wills *et al.*, 2005) and UL16 (compare section 1-6). HLA-E expression of cells depends on the binding of a peptide derived from other MHC class I molecules, which normally prevents NK cell recognition by binding to its inhibitory CD94/NKG2A receptor. MHC class I downregulation of virus infected cells would result in recognition by NK cells. CMV UL40 product, however, was shown to substitute for this peptide to protect infected cells (Tomasec *et al.*, 2000) although this was not confirmed in a subsequent study (Falk *et al.*, 2002). The product of UL18 mentioned earlier was also reported to inhibit NK cell lysis in some studies (Reyburn *et al.*, 1997) but was shown to have a NK cell activating effect in others (Leong *et al.*, 1998). A more recent study demonstrated interaction of the UL83 encoded protein pp65 with an activating NK cell receptor that is thought to represent yet another strategy to reduce NK cell mediated killing (Arnon *et al.*, 2005). Furthermore CMV was demonstrated to encode cellular receptors for the Fc

domain of IgG (UL118/119 and TRL11/IRL11), the function of which is not yet determined (Atalay *et al.*, 2002) but may involve viral immune escape from complement (compare section 1-6). The protein pp65 was shown to decrease expression of HLA class II molecules in infected cells (Odeberg *et al.*, 2003) and phosphorylate the IE1 encoded 72 kDa protein, thereby interfering with its processing and presentation for immunosurveillance by $CD8^+$ T cells (Gilbert *et al.*, 1996).

There also exists circumstantial evidence for CMV escape from IFNγ responses by the loss of infected cells to respond to IFNγ. Lucin and colleagues found that in cells treated with the cytokine before CMV infection, IFNγ, in synergism with TNFα, blocked late CMV gene transcription, reduced viral DNA replication and altered nucleocapsid formation. However, simultaneous infection and cytokine treatment did not result in a comparable effect (Lucin *et al.*, 1994). Similar observations were made during IFNγ's effect on antigen presentation in cells exposed to IFN γ before CMV infection but not during simultaneous infection and cytokine treatment (Hengel *et al.*, 1995). Zimmermann and colleagues demonstrated that IFNγ receptor signal transduction was affected by the product of the murine CMV m27 gene (Zimmermann *et al.*, 2005). Since a human gene homologous to m27 exists (Chee *et al.*, 1990), it may exert a similar function during human CMV infection.

Although immune evasion strategies of CMV ensure survival of the virus in its host, CMV specific $CD8^+$ T cells remain critical in controlling viral replication and immunogenicity of several proteins has been demonstrated (Sylwester *et al.*, 2005). The principle of restoring the highly balanced relationship (that exists between CMV and immunocompetent individuals) in immunocompromised individuals by the transfer of CMV specific memory $CD8^+$ T cells was proven (Walter *et al.*, 1995). For studies in that area accurate measurement of such cells *in vivo* is crucial.

1-10 Quantification of CMV specific $CD8^+$ T cells with HLA/peptide tetramers

Different viral $CD8^+$ T cells can mediate control of virus infections. Clonal competition occurs (compare section 1-11.2), which is thought to differ substantially in a lymphopenic environment (such as in HSCT patients) from that in healthy individuals with normally balanced haematopoiesis (Goldrath and Bevan, 1999, Goldrath *et al.*, 2000). Approaches used for quantifying the different specific $CD8^+$ T cells traditionally measure the function of those cells.

The limiting dilution assay (LDA) can be used to define an unknown frequency of effector T cells within PBMCs based on a functional read-out that is usually proliferation. It is a dose-response assay that allows detection of either positive or negative response to antigen in each individual culture within replicates. Several culture replicates that vary in the number of PBMC are tested. Entirely positive cultures are not informative because it is unknown whether one or more precursor cells in the culture responded. However cultures with negative responses in one or several replicates demonstrate that there are no precursors of the tested specificity in at least some replicates of that culture. Therefore it is possible to evaluate the effector T cell frequency in the original PBMC population by determining the number of cultures that are negative. To achieve this the fraction of negative cultures of replicates of a given number of PBMC can be used in suitable statistical tests including regression analysis and the least square method (Moretta *et al.*, 1983).

LDA can also be based on a chromium release assay used to determine the cytotoxic potential of $CD8^+$ T cells. This assay uses the ^{51}Cr isotope of chromium for labelling of target cells and is based on the measurement of membrane disintegration through leakage of cytoplasm. $CD8^+$ T cells are added and tested for their cytolytic function towards target cells whereby the amount of ^{51}Cr released into the supernatant is proportional to the number of targets killed (McCoy *et al.*, 1973). Alternatively LDA can be based on DNA fragmentation occurring early (before membrane disintegration) during apoptosis after $CD8^+$ T cell induced cell death. Therefore target cells are labelled with the radiochemical tritiated thymidine ([^{3}H] TdR) during growth phase. This results in incorporation of [^{3}H] TdR into the DNA of target cells. They are then incubated with graded numbers of $CD8^+$ T cells. Cells are harvested on glass filters whereby fragmented DNA from dead cells is washed through and the DNA retained by living cells is measured by radioactivity (Matzinger, 1991).

The major drawback of the traditional LDA method (besides requiring large numbers of cells) is the failure to detect cells that are not functional *in vitro*, which can lead to underestimation of specific T cells (Goulder *et al.*, 2000, Moss *et al.*, 1995). CMV specific $CD8^+$ T cell frequencies were measured directly using HLA/peptide tetramers in this project. Their functionality was tested secondarily by the detection of cytokine release upon stimulation with antigen. The basics underlying the measurement with HLA/peptide tetramers are briefly described below since they represent the platform for the main read-outs that will be described in the result chapters of this thesis.

The development of MHC class I tetramer/peptide tetramer complexes (tetramers) in 1996 represents the first direct method for measuring frequencies of antigen-specific $CD8^+$ T cells (Altman *et al.*, 1996). Tetramers are multimeric forms of complexes of human leukocyte antigen (HLA) class I molecules and specific antigenic peptides conjugated to fluorochromes as illustrated in Figure 1-12.

Figure 1-12 Model of an HLA/peptide tetramer complex

This figure represents a backbone tube representation of an HLA/peptide tetramer complex (compare with MHC/peptide monomer representation in Figure 1-8 on page 65). Streptavidin is shown in light blue, the fluorochrome (here phycoerythrin) in red. HLA heavy chain is represented in dark blue, ß2m in intermediate blue and peptide in purple (adapted from (McMichael and O'Callaghan, 1998)).

Binding of single HLA/peptide complexes with TCR is of low affinity and therefore has a fast dissociation rate (reviewed in (Davis *et al.*, 1998)). Boniface and colleagues demonstrated that ligand driven formation of TCR clusters is required and therefore three or more TCR need to be engaged to initiate effective activation of $CD8^+$ T cells (Boniface *et al.*, 1998). HLA/peptide tetramers are produced by multimerisation with streptavidin. Since streptavidin has a tetrahedral symmetry it is likely that only three HLA/peptide complexes may engage TCR on the surface of a $CD8^+$ T cell at any one time. Furthermore the most commonly used fluorochrome phycoerythrin is quite large (compare Figure 1-12) and likely to interfere with access of one HLA/peptide complex and therefore again binding is likely to be trimeric (reviewed in (McMichael and

O'Callaghan, 1998)). The multimers provide multiple ligands for enhanced interaction with antigen specific $CD8^+$ T cells. Therefore they have an increased overall avidity and a slower dissociation rate than monomers, which makes them more suitable for immunological staining of $CD8^+$ T cells. The generation of HLA/peptide tetramers accounts for a major part of this project. It did involve the production of HLA heavy chain and $ß_2m$ proteins from expression vectors, followed by refolding with peptide and multimerisation. Production steps were optimised for the different HLA/peptide combinations. Details on this will be described in section 2-11.

Binding of these complexes to the TCRα/ß on $CD8^+$ T cells (compare Figure 1-8, page 65) is highly specific (Burrows *et al.*, 2000) provided that an optimum staining temperature is used (section 2-5.1). They can be used to directly visualise and enumerate antigen-specific $CD8^+$ T cells by flow cytometry (section 2-5.3) without the requirement for *in vitro* stimulation and have made a major contribution towards the study of T cells demonstrating the importance of the $CD8^+$ T cell immune defence against CMV.

It was suggested that the intensity of T cell staining obtained by TCR binding of tetramers is proportional to the avidity of the antigen specific T cells, which raises the possibility to use the technique to purify high avidity (and therefore highly reactive) antigen specific T cells (Yee *et al.*, 1999). High avidity antigen specific T cells from donors obtained by tetramers may then be transferred to patients for immunotherapy.

1-11 Adoptive therapy with CMV specific T cells post HSCT

As described in detail in the earlier sections of this chapter, CMV infection is a major problem in HSCT patients. Conditioning regimens eliminate the recipient immune defence. T cell depletion, delayed T cell recovery *in vivo* and prolonged immunosuppressive treatments are associated with a significant risk of viral reactivation post HSCT. Vaccination approaches have been developed, which aim to evoke an immune response by administration of an immunogen to individuals. These initially made use of whole live virus but more recently involved the antibody target glycoprotein B as well as dense bodies and T cell targets, which are either pp65 and IE proteins administered in expression vectors or minimal epitopes derived from these proteins (reviewed in (Wills *et al.*, 2002a)). However, these vaccination approaches may not function optimally in immunocompromised hosts. Antiviral drugs inhibit productive virus replication *in vivo*. However, they appear to alter subsequent reactivations such that antiviral T cells recover less effectively. This can lead to late, chronic CMV reactivation, particularly in association with chronic GvHD (Junghanss *et al.*, 2002, Nguyen *et al.*, 1999). In addition, drug resistant escape mutants have been reported (Springer *et al.*, 2005) and the treatment itself is associated with significant toxicity in patients. Adoptive therapy of donor derived CMV specific $CD8^+$ T cells is a promising treatment alternative (Gandhi *et al.*, 2003b).

Reusser and colleagues demonstrated that cytotoxic activity of CMV specific $CD8^+$ T cells against infected fibroblasts correlates with resistance against CMV pneumonitis (Reusser *et al.*, 1991). A proof of principal for the feasibility of an adoptive immunotherapy approach against CMV in transplant patients has been established in a phase 1 clinical trial by Walter and colleagues (Walter *et al.*, 1995), who isolated and expanded CMV specific $CD8^+$ T cell clones from the blood of HSCT donors and administered them to 14 patients prophylactically at weekly intervals in doses escalating from $3.3x10^6$ to $1x10^9$/kg beginning at 30 to 40 days post transplant. Their results strongly suggest a safe, protective effect against CMV reactivation. Neither CMV viraemia nor disease developed in any of the treated patients. $CD8^+$ T cell responses declined over some weeks in patients who failed to recover endogenous CMV specific $CD4^+$ T cell responses suggesting that $CD4^+$ T cells are required for the persistence of transferred $CD8^+$ T cells. However, it has to be noted that the respective patients had GvHD related treatment with cyclosporine and prednisolone. Therefore it is not known if the decline in transferred $CD8^+$ T cells was due to $CD4^+$ T cell deficiency

or the immunosuppressive treatment. Einsele and colleagues (Einsele *et al.*, 2002) published a second immunotherapy trial. They infused polyclonal CMV specific T cell lines into eight HSCT recipients who had persisting or recurring CMV infection despite the prolonged used of antiviral treatment. Seven of these patients still received immunosuppressive GvHD prophylaxis at the time of first cell infusions. Cell lines were prepared by stimulation of PBMC with CMV lysate and expanded with autologous, irradiated PBMC feeder cells for presentation of CMV lysate and IL2. They were predominately $CD4^+$. A total dose of $1x10^7/m^2$ was administered with no cytotoxicity observed. Viral load was subsequently reduced in 7/7 evaluable patients. This reduction was persistent in five and transient in two patients, who both had received intense immunosuppression for GvHD around the time of T cell therapy. A similar method was also successfully employed by Peggs and colleagues (Peggs and Mackinnon, 2004a, Peggs *et al.*, 2003c).

Despite the positive outcome of both these trials, there were some associated difficulties. The methodology by Walter and colleagues required skin biopsy for the generation of fibroblasts (that were used as APC) and live virus to stimulate T cells. The method by Einsele and colleagues required labour intensive *in vitro* expansion of cells. The feasibility of a more rapid expansion method using a 20-hour incubation period of donor PBMC with pp65 and subsequent isolation of IFNγ producing T cells was demonstrated by Mackinnon and colleagues. Adoptive transfer of $1x10^4$ cells/kg generated with this method either prophylactically (n=2) or pre-emptively (n=5) resulted in 700 to 5000 fold *in vivo* expansion and significant reduction of CMV reactivation periods in treated patients (Mackinnon *et al.*, 2007).

The correlation of CMV specific $CD8^+$ T cells with protection against CMV disease in HSCT patients was subsequently confirmed by quantification using tetramers (Aubert *et al.*, 2001, Cwynarski *et al.*, 2001, Gratama *et al.*, 2004, Gratama *et al.*, 2001). Therefore CMV peptide specific $CD8^+$ T cells that can be detected and sorted by tetramers could be used for an adoptive therapy in immunocompromised patients. This may result in highly specific protection against CMV without the use of live virus or usage of a highly labour intensive tissue culture based system. Direct tetramer based selection may be advantageous over the use of *in vitro* cultured T cells since it is unclear what effect the manipulations may have on their cellular function. Indeed, the direct application of such cells without prior *in vitro* expansion has been demonstrated in a phase 1 clinical trial (Cobbold *et al.*, 2005).

For the development of an adaptive therapy approach, epitopes targeted by $CD8^+$ T cells in an HLA dependent context as well as the number of specific T cells necessary for a protective response *in vivo* must be known. Initial CMV T cell studies addressing these questions focused primarily on frequency measurements of pp65 specific $CD8^+$ T cells restricted by HLA-A*02 and HLA-B*07 because these alleles are amongst the most common HLA types in Caucasoids and many other ethnic groups. The successful generation of HLA-A*0201 restricted CMV pp65 (495-503) specific $CD8^+$ T cells under GMP conditions has already been demonstrated (Li Pira *et al.*, 2006) It is, however, desirable to develop reagents for an adoptive therapy approach directed towards different epitopes in the context of more than two HLA alleles, as this could benefit more patients.

1-11.1 T cell targets: HLA candidates and their frequency in the population

HLA/peptide tetramers are highly specific and their use is limited to individuals possessing T cells, which have been selected to react with peptides presented by relevant self-HLA molecules. Optimal therapeutic application may be achieved by the selection of tetramers comprising a wide variety of HLA alleles that are relatively common in most ethnic populations. Previous studies suggested that 90 % coverage of some ethnic groups might be attainable with as few as eleven $CD8^+$ T cell epitopes (Longmate *et al.*, 2001).

According to Marsh, HLA-A2 has a gene frequency of 25 % in Caucasoids (Marsh, 2000). Longmate and colleagues estimated that the most common HLA-A2 allele, HLA-A*0201, covers 45.8 % of European Caucasoids (Longmate *et al.*, 2001). To choose further HLA alleles that may allow the most possible coverage of Caucasoids, gene frequencies of the most common (>8 %) HLA genes within this ethnic group were compared and are shown in Table 1-1.

HLA-A	Gene Frequency
HLA-A2	25.0 %
HLA-A1	14.0 %
HLA-A3	11.9 %
HLA-A24	10.4 %
HLA-B	**Gene Frequency**
HLA-B44	11.2 %
HLA-B35	10.3 %
HLA-B7	8.7 %

Table 1-1 Average gene frequency of the most common HLA-A and HLA-B genes amongst Caucasoids

This table demonstrates average gene frequencies of HLA genes that are most common amongst Caucasoids (Marsh, 2000).

This table demonstrates that besides tetramers that incorporate the well-studied HLA-A2 or HLA-B7, it would be desirable to investigate CMV specific $CD8^+$ T cell responses with tetramers that incorporate HLA-A1, HLA-A3, HLA-A24, HLA-B44 or HLA-B35. Accordingly efforts during this project were focused on the generation of tetramers comprising the most common alleles of these less well-studied HLA genes.

An HLA-A*0301 restricted CMV pp65 epitope was not known at the time of initiating this project. Enzyme-linked immunospot (ELISpot) based investigations of the HLA-A*0301 restricted CMV pp150 (945-955) $CD8^+$ T cell epitope defined by (La Rosa *et al.*, 2005) yielded very low or undetectable responses in healthy individuals. Therefore investigations of HLA-A*0301/CMV specific $CD8^+$ T cells had to be halted to prioritise investigations with tetramers that showed more promising production developments first.

HLA-B*4402 encoded protein was successfully produced by cloning from homozygous cell lines and expression in *Escherichia coli (E. coli)*. However, refolding (as is necessary for the production of HLA-B*4402/CMV tetramer) proved to be difficult and after lengthy, unsuccessful trials using alternative refolding strategies, investigations of HLA-B*4402/CMV specific $CD8^+$ T cells had to be halted as well to prioritise investigations with tetramers that showed more promising production developments first.

Tetramers incorporating HLA-A*0101, HLA-A*2402 or HLA-B*3501 and relevant CMV peptides were produced successfully. This involved several optimisation strategies of the original method described by Altman and colleagues (Altman *et al.*, 1996) and took up a major part of this project. Results obtained during the generation of

these tetramers will be described in Chapter 2. Findings from the measurement of T cell responses obtained with these reagents will subsequently be shown in the result chapters of this thesis.

HLA-A*2402 is of particular interest regarding coverage of multiple ethnic populations because it is the most frequent HLA allele in the Japanese population. The allele frequencies of HLA-A*0201 and HLA-B*0702 in this population are only 11 % and 5 % respectively whereas the allele frequency of HLA-A*2402 is 32.7 % in the Japanese (Tokunaga *et al.*, 1997). Sette and Sidney estimated that the HLA-A*2402 allele covers 58 % of the Japanese (Sette and Sidney, 1999). Longmate and colleagues estimate that successive addition of HLA-A*0201, HLA-A*2402 and HLA-A*0101 already covers 75.7 % of the Caucasoid population. Accordingly adoptive transfer of $CD8^+$ T cells targeting CMV peptides presented by the common HLA-A*0101, HLA-A*2402 and also HLA-B*3501 in addition to HLA-A*0201 and HLA-B*0702 may enable therapy of a majority of European Caucasoids and Asians. Consistent with that, this project aimed to further the knowledge on the less well-studied HLA-A*0101, HLA-A*0201, HLA-A*2402 and HLA-B*3501 restricted CMV specific $CD8^+$ T cells, which may be most relevant for inclusion in future adoptive therapy trials.

1-11.2 T cell targets: CMV peptide candidates and their immunogenicity

Of all the peptides encoded by a foreign antigen such as CMV that can potentially be presented to $CD8^+$ T cells, only a small proportion induces measurable responses in association with any given HLA allele. This observation is known as immunodominance. Yewdell and Bennink reviewed that to be immunogenic; peptides must first be able to bind to an HLA class I allomorph (the product of an HLA class I allelic gene) with high enough affinity. This is estimated to narrow down the number of potential peptides by 1/200. Then limitations in the $CD8^+$ T cell repertoire render 1/2 of the HLA binding peptides non immunogenic. Of these, limitations in the efficiency of antigen processing render a further 4/5 non immunogenic (Yewdell and Bennink, 1999). The competition for limited resources between $CD8^+$ T cells responding to the remaining immunogenic peptides (0.2 % of peptides encoded by the foreign antigen) finally dictates the outcome of the overall T cell response in the host.

Kedl and colleagues reviewed that T cells may compete when the number of APC or antigen is limited and the T cells are present in numbers high enough to influence one another. Competition appears to primarily occur between $CD8^+$ T cells

responding to the same HLA/antigen (direct competition) rather than between $CD8^+$ T cells responding to different HLA/antigens (cross competition) (Kedl *et al.*, 2003). Direct competition may result from either high affinity T cells removing HLA/antigen from the APC (shown in Drosophila (Huang *et al.*, 1999)), thereby decreasing the chance of lower affinity T cells to detect it or from killing of APC (demonstrated for infection with *Listeria monocytogenes* (Wong and Pamer, 2003)). These mechanisms can explain affinity maturation of $CD8^+$ T cells. Internalisation of HLA/peptides by T cells in turn results in themselves becoming sensitive to lysis by T cells specific for the peptides and may serve to down-regulate immune responses (Huang *et al.*, 1999). Indirect competition likely results from the limitations of the space surrounding APC and plays a role in driving epitope dominance (reviewed in (Kedl *et al.*, 2003)).

The CMV genome contains more than 200 open reading frames (Chee *et al.*, 1990). At the time of initiating this project, evidence suggested that the major tegument protein pp65 and the immediate early protein IE1 are the main immunodominant targets for $CD4^+$ and $CD8^+$ T cells (Beninga *et al.*, 1995, Boppana and Britt, 1996, Davignon *et al.*, 1995, Kern *et al.*, 2002, Kern *et al.*, 1999b, Wills *et al.*, 1996). Since the quantity of pp65 specific $CD8^+$ T cells correlating with protection from CMV disease were previously determined in the context of HLA-A*0201, this study aimed to compare $CD8^+$ T cell responses recognising pp65 presented by this and other common HLA alleles (initially HLA-B*3501 and HLA-A*2402) and to investigate the correlation of those cells with CMV replication in HSCT patients.

During the development of this project, a peptide derived from pp50 was shown to induce HLA-A*0101 restricted $CD8^+$ T cell responses of a magnitude similar to those observed for pp65 and IE1 for the first time (Elkington *et al.*, 2003). At this time the panel of CMV specific $CD8^+$ T cell responses to be investigated was expanded to include HLA-A*0101 restricted $CD8^+$ T cell responses targeting pp65 and also the new pp50 epitope.

High magnitudes of a focused response targeting mainly two proteins seem surprising considering pp65 is one amongst a large number of proteins encoded by the CMV genome. Accordingly a recent report demonstrated new data revealing a much broader specificity of $CD8^+$ T cell responses (Sylwester *et al.*, 2005). Knowledge on the two protein targets, pp65 and pp50, which were investigated in this study, is summarised below.

The CMV tegument phosphoprotein, pp65, is considered one of the most important antigens for cellular immunity in CMV infection (McLaughlin-Taylor *et al.*, 1994, Walter *et al.*, 1995). This structural protein is introduced into the cell after viral penetration. Therefore recognition can occur in the absence of *de novo* viral gene expression. $CD8^+$ T cells targeting pp65 are thought to lyse infected cells before viral assembly and may provide a biological advantage for limiting the spread of infection after CMV reactivation (Riddell *et al.*, 1991a). Dense bodies were shown to consist mainly of pp65 (Irmiere and Gibson, 1983) and to enter cells efficiently, delivering protein into the cells and eliciting $CD8^+$ T cell responses in the absence of *de novo* protein synthesis (Pepperl *et al.*, 2000). The inhibition of protein presentation due to downregulation of class I MHC as one of the immune escape strategies of CMV might further explain why the specificity of the $CD8^+$ T cell responses seems to be focused on pp65 (Beersma *et al.*, 1993, Gilbert *et al.*, 1993, Gilbert *et al.*, 1996, Spaete *et al.*, 1994, Warren *et al.*, 1994). Wills and colleagues demonstrated that the $CD8^+$ T cell response to CMV is dominated by pp65 irrespective of major histocompatibility complex haplotype. The group used a pp65 gene deletion mutant of CMV to show that $CD8^+$ T cells targeting this protein contribute to 70 to 90 % of all $CD8^+$ T cells recognising CMV infected cells (Wills *et al.*, 1996). Kern and colleagues found that over 80 % of normal healthy donors have $CD8^+$ T cell responses to peptides derived from pp65 (Kern *et al.*, 2002).

The importance of CMV antigens such as pp50 may have been underestimated prior to 2003. Profiling of $CD8^+$ T cell responses to a large panel of CMV antigens during that year revealed, for the first time, that more than 40 % of $CD8^+$ T cell responses to CMV are directed towards antigens other than pp65 or the immediate early protein 1 (Elkington *et al.*, 2003).

In contrast to pp65, pp50 is a non-structural protein. Alternative names of this phosphoprotein are pUL44 and p52. At present the knowledge on this protein is much more limited than that on pp65. It is known, together with pUL54, to form the viral-encoded DNA polymerase complex that mediates replication of CMV in the nucleus of infected cells. More recently it was demonstrated to also form disulfide-linked homopolymers in the cytoplasm of cells (Alvisi *et al.*, 2006). It was reported to be released from cells upon cell lysis of infected cells and to aid cell adhesion during viral entry by binding to integrins on target cells in mice (Loh *et al.*, 2000). It is one of the most immunogenic CMV proteins that can induce a humoral response (Gergely *et al.*,

1988). The promoter of the gene encoding CMV pp50 was shown to use three transcriptional start sites that were expressed differentially throughout infection (Leach and Mocarski, 1989). Therefore translation starts early (4 hours post infection) but maximal synthesis occurs late (48 hours) after infections (Geballe *et al.*, 1986). Since viral immunomodulating proteins are known to block endogenous antigen presentation by CMV infected cells, CMV pp50 specific $CD8^+$ T cells may also be induced primarily by cross priming (compare section 1-6). In comparison, it was demonstrated that for many EBV-encoded antigens, which are poorly processed by infected cells, $CD8^+$ T cells are generated through the cross-priming pathway (Blake *et al.*, 2000, Blake *et al.*, 1997).

Amino acid sequences of pp65 and pp50 derived epitopes, which were targets of CMV specific $CD8^+$ T cells studied during this project, are summarised in Table 2-5 on page 129. Relevant publications on details and immunological significance of these epitopes are introduced during the relevant sections in Chapter 3.

1-12 Aims of this thesis

Current limitations of antiviral therapies identify a need for alternative approaches, such as an adoptive therapy, to combat the development of potentially fatal CMV related complications in HSCT patients. Tetramers are a valuable tool for highly specific selection and thereby therapeutic use of antigen specific T cells and for monitoring of those cells *ex vivo*.

The study described in this thesis aims to further the knowledge firstly on the quantity of CMV specific $CD8^+$ T cells that correlate with protection from CMV replication *in vivo* and secondly on the fine specificity of those cells.

The aim of this project was to investigate CMV specific $CD8^+$ T cell responses restricted by common HLA types other than the well studied HLA-A2 and HLA-B7 types. Investigations covered responses to CMV peptides restricted by some of the most frequent HLA alleles amongst Caucasoid and Asian ethnic groups. This involved the development of corresponding tetramers to enumerate $CD8^+$ T cells that target CMV in patients who reconstituted these responses after severe immunosuppression due to conditioning for HSCT. Longitudinal measurement of CMV specific $CD8^+$ T cells was correlated with clinical data. The cells investigated were not studied with the same frequency and confirmed functionality in this patient group before, and this was the first study to address the question of which number of these cells (inversely) correlated with the ability to detect CMV replication *in vivo*. The results have direct implications for the development of CMV specific adoptive therapy in this patient group.

The different CMV specific $CD8^+$ T cells were analysed for their TCR diversity directly *ex vivo*. This study represents the first to describe TCR diversity of $CD8^+$ T cells targeting a CMV pp50 epitope in comparison to different CMV pp65 epitopes. This analysis may aid to determine which CMV specific $CD8^+$ T cells are most likely to efficiently protect and benefit patients from amongst those targeting different CMV epitopes. CMV specific $CD8^+$ T cells with high T cell receptor diversity may counter immune escape mechanisms and contribute only minimally to CMV associated immunosenescence, thereby providing an advantage over epitope specific $CD8^+$ T cells with less T cell receptor diversity.

CHAPTER 2 MATERIALS AND METHODS

2-1 Introduction

The study described in this thesis primarily focuses on investigations involving the use of different tetrameric complexes and spectratyping analysis. Especially the generation of tetramers needed for the measurement of T cell responses accounts for a major part of the PhD project. Consistent with that the description of materials and methods in this chapter contains a main section that describes details of how the different tetramers were generated, including cloning of expression vectors, followed by expression, refolding and tetramerisation of proteins. Results obtained during the generation of tetramer reagents are included within this chapter rather than in the results chapters to draw the reader's attention to the measurement of patient T cell responses rather than to preliminary experiments in the result chapters. In contrast, optimisation of spectratyping methods is described in the relevant result chapter to highlight new settings that were developed to enable spectratyping of small numbers of *ex vivo* PBMC.

All materials and methods used to perform the work mentioned throughout this thesis are described in this chapter. Buffer and solutions were prepared using chemicals purchased from BDH or Sigma if not mentioned otherwise. These were filter sterilised using 0.2 μm filters or autoclaved if their properties allowed so.

2-2 Patients & healthy donors

Patients and healthy donors were recruited for this study if they expressed the HLA types HLA-A*0101, HLA-A*0201, HLA-A*2402 or HLA-B*3501 (subsequently abbreviated as HLA-A1/A2/A24/B35) and they and/or their donors were CMV seropositive. A minority of healthy donors, some of them with unknown HLA type and/or CMV serology, were recruited for control experiments. All subjects gave informed consent (see section 2-2.2).

2-2.1 Determination of HLA type and CMV serology

The majority of DNA-based HLA typing assays for patients and healthy donors were performed at the Histocompatibility Laboratories at the Anthony Nolan Research Institute (ANRI). HLA class I genotyping was performed using sequence-specific oligonucleotides (PCR-SSO), sequence-specific primers (PCR-SSP), or nucleotide sequencing. PCR-SSO involves an HLA locus specific polymerase chain reaction

(PCR). The product is denatured and hybridised against a panel of immobilised oligonucleotide probes that have sequence complementary to stretches of polymorphisms within HLA alleles. The presence of bound amplicon is measured in a colour reaction. The PCR-SSO methodology provides low to medium resolution results usually at the two-digit level. PCR-SSP and nucleotide sequencing analysis can provide high-resolution allelic level typing results represented by four digits. PCR-SSP involves multiple PCRs targeting the presence and absence of polymorphisms within HLA genes. Protocols usually also include internal controls from constitutive genes and products are analysed by agarose gel electrophoresis and staining. Depending on the primers used PCR-SSP can initially provide low to medium resolution typing. Additional testing after the low-resolution tissue type is known, can give a high-resolution result. PCR-SSP is the quickest DNA based method. However, it is not used for large scale HLA typing because of the rate-limiting step of agarose gel electrophoresis. Therefore the Histocompatibility Laboratories at the ANRI use a combination of both methods and nucleotide sequencing to discover novel mutations.

Patients that had not been HLA typed routinely at the high-resolution level before transplantation (for example patients scheduled for autologous transplantation) and healthy donors were typed for the major HLA antigens (HLA-A and HLA-B, HLA-C, HLA-DR, HLA-DQ and HLA-DP, compare section 1-2) at the allelic level at the Histocompatibility Laboratories at the ANRI.

Previous CMV infection was detected by the presence of specific G isotype Ig in the serum. The additional presence of specific M isotype Ig was also tested for and will be described later. The majority of CMV serology assays for patients and healthy donors were performed at the Histocompatibility Laboratories at the ANRI using the Bioelisa CMV IgG kit (Biokit Ltd).

This enzyme-linked immunosorbent assay (ELISA) test was used for detection of IgG antibodies to CMV in heat-inactivated serum. Briefly, serum was incubated in microplate wells (U 69 Maxisorb Immunoplate, Nunc) coated with CMV antigen. CMV antibodies, if present, then bound to the antigens attached to the well. The well was washed to remove residual serum and enzyme-labelled antibodies that bind to human IgG (rabbit anti-human IgG conjugated with peroxidase) were added. After another wash to eliminate unbound material, an enzyme substrate solution (citrate-acetate buffer containing hydrogen peroxide) containing a chromogen (3,3', 5,5'-Tetramethylbenzidine dissolved in dimethylsulfoxide) was added and developed a blue colour if the serum

contained anti-CMV IgG. The blue colour was converted to yellow by addition of sulphuric acid stop solution. The intensity of the colour was proportional to the amount of anti-CMV IgG in the test serum and was estimated by measurement at 450 nm along with a calibration curve.

Early infection resulting in the presence of IgM but not IgG antibodies to CMV would be missed by the Bioelisa CMV IgG kit. Therefore CMV IgM serology was additionally tested using the Bioelisa CMV Colour (Biokit Ltd). In comparison to the IgG kit, this is a qualitative assay using a conjugate that is based on a mixture of anti-human IgG and anti-human IgM. It should be noted that CMV IgM antibody can be present in patients with reactivation of latent CMV (Pass *et al.*, 1983) and therefore cannot be used to characterise primary infection (Subramanian, 2008).

Patients 10 and 15 were HLA typed (PCR-SSO in both cases) and tested for CMV (by fluorescent anti-pp65 and anti-pp67 antibody, and ELISA respectively) serology locally at the Universidad Autónoma de Madrid (UAdM) in Madrid, Spain and the Universidade Federal do Paraná (UFdP) in Curitiba, Brazil respectively.

2-2.2 Ethical approval

Recruited patients gave informed written consent to participate in the study that was ethically approved by local authorities at the Royal Free Hospital (RFH) in London, and for consultation of their clinical data.

Patients were approached individually by the treating hospital's medical staff, who informed them about the objectives and protocol of the study. Each patient was given an information sheet, which was translated into the patient's native language when necessary. Patients who decided to enter the study were asked to sign a consent form.

2-2.3 Blood sample collection

Blood was collected from patients at the Haematology Day Unit (which was initially located at Crowley Ward and later at OACS (Oaks Ambulatory Care Suite)) at the RFH, London; the UAdM (in case of patient 10); the UFdP (in case of patient 15) or from healthy volunteers working at laboratories at the ANRI or at the Haematology and Virology Departments of the RFH after informed consent. Additional blood samples from patient 2 were also obtained from the Royal Devon & Exeter Hospital when the patient had moved away from London.

For patients, 20 ml of peripheral blood samples were drawn in parallel with blood being taken for routine tests required for assessment of the progress of patients at visits to the clinic and therefore did not require additional venipuncture.

Blood samples obtained from the RFH were drawn into tubes containing 20 µl of Heparin (Monoparin 1000 U/ml, CP Pharmaceuticals) to prevent coagulation and were processed the same day. Blood samples obtained from the UAdM were drawn into tubes containing transport medium of a volume equal to that of the blood sample. Transport medium consisted of sterile filtered Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (BioWhittaker) supplemented with trisodium citrate at a final concentration of 3.3 % (w/v) and ß-mercaptoethanol (BDH Biochemicals) at a final concentration of 5 µM. Blood samples preserved in that way were shipped by overnight delivery service and process at the ANRI the following day. Blood samples obtained from the UFdP were processed locally. Frozen PBMC were brought by collaborators on their way to London and stored in liquid nitrogen until used for experiments at the ANRI.

2-2.4 Consultation of clinical data including CMV PCR

Clinical data was consulted with the help of medical staff at the treating hospitals. This included general patient information, type of transplant received, conditioning regimens and potential T cell depletion of the grafts, T cell counts determined at the time of sampling, CMV DNAemia/antigenaemia results, antiviral treatment, GvHD prophylaxis, GvHD episodes and administration of immunosuppressive treatments. Complete information on the last three mentioned parameters could not be obtained for patients 1 and 19 because clinical data from these private patients had been removed from the RFH and access was not provided at the new location.

CMV DNAemia was assessed from whole blood by quantitative PCR performed at the Virology Department of the RFH as described in (Mattes *et al.*, 2005) or, in case of patient 10, at the UAdM. For patient 15, CMV was detected by antigenaemia using perinuclear staining of leukocytes indicating the number of CMV infected cells per 200,000 PBMC (van der Bij *et al.*, 1988) stained during routine viral monitoring by the Virology Department of the UFdP.

2-2.5 Patient's and healthy donor's characteristics

All patients were undergoing haematopoietic stem cell transplantation (HSCT) for haematological conditions and had been HLA typed at the allelic level by molecular techniques.

Patients from whom samples were collected from earlier than 100 days post transplantation onwards with at least 4 collections post transplantation were included in the follow-up cohort to establish a threshold of cell levels correlating with protection from CMV. Characteristics of these patients are demonstrated in Table 2-1. They were monitored weekly from engraftment on until discharge from hospital and at visits to the clinic thereafter.

Most patients that were scheduled for allogeneic HSCT (all allogeneic patients shown in Table 2-1 except patients 6, 10, 17, 22, 24 and 26, who did not receive CMV prophylaxis and patient 15 described later) received antiviral prophylaxis in form of intravenous aciclovir at 10 mg/kg of body weight, 3 times a day, during the in-patient stay and were given 2 g/kg of body weight oral valaciclovir, four times a day, thereafter. Aciclovir or valaciclovir were also given prophylactically to these patients between courses of antiviral treatment and continued until at least five months post transplantation. Patient 15, who was transplanted at the UFdP, received intravenous aciclovir at 250 mg/m^2 (height = 1,66 m, weight = 63,8 kg, total corporal surface = 1,71 m^2), 3 times a day from day -1 to +28 post transplantation. GvHD prophylaxis, usually in form of Cyclosporine A alone or with Methotrexate, that was given to patients are listed in Table 2-1. Most patients that were scheduled for autologous HSCT (all autologous patients shown in Table 2-1 except patients 14, who did not receive CMV prophylaxis) received the same CMV prophylaxis as that described above as in-patients, which was then stopped on discharge. Patients transplanted after renewal of these prophylaxis guidelines at the RFH (patients 6, 14, 17, 22, 24 and 26) or patient 10, who was transplanted at the UAdM did not receive CMV prophylaxis.

Antiviral and immunosuppressive treatment received in addition to prophylaxis by the patients is shown in the follow-up graphs in the result chapters. Antiviral treatment usually consisted of intravenous ganciclovir at 5 mg/kg of body weight twice a day (simultaneously with subcutaneous intravenous granulocyte colony-stimulating factor (G-CSF) at 300 mg/kg of body weight once daily or on alternate days to counteract its myelosuppressive effect) or intravenous foscarnet at 90 mg/kg of body

weight twice a day. Patients transplanted after renewal of these treatment guidelines (all patients shown in Table 2-1 except patients 1, 9, 10, 15, 16 and 19) were also eligible for alternative treatment with oral Valganciclovir at 900 mg/kg of body weight twice a day. Doses were reduced if patients suffered renal impairment. Pre-emptive treatment in patients 1, 9, 16 and 19 was initiated after two consecutive positive CMV PCR results and until two consecutive negative CMV PCR were observed. In patients transplanted after renewal of these treatment guidelines (all patients shown in Table 2-1 except patients 1, 9, 10, 15, 16 and 19) treatment was warranted if the viral load was ≥3,000 genomes/ml. Patients were randomised to be treated either until two consecutive negative CMV PCR were observed (all patients except patients 10, 15 and 23) or until the viral load was ≤3,000 genomes/ml on two consecutive occasions (patient 23). Patients 10 and 15 did not receive treatment according to these guidelines because they received HSCT at the UAdM and UFdP respectively. Antiviral and immunosuppressive treatment received by these patients is shown in the follow-up graphs in the result chapters.

Immunosuppressive treatment usually consisted of methylprednisolone and was given at a dose and time period appropriate for the severity of GvHD symptoms. The type and time period of treatment received is shown in the result chapters.

Patients, who did not meet the criteria to be included in the follow-up cohort mentioned earlier, but from whom samples were used for clonotypic and other experiments that are shown throughout this thesis, are listed in Table 2-2.

Healthy donors from whom measurements are shown throughout this thesis are listed in Table 2-3.

Patient ID	HLA of interest	Diagnosis prior to HSCT	Sex (r/d)	Age (r/d)	CMV serol. (r/d)	HSC source****	Conditioning	TCD of graft	GvHD prophylaxis	CMV disease*	Sample no.**	Period ***
1	A2, B35	AML (tr2)	m/f	43/41	+/+	m allo PBSC M sib	Bu, Cam, Flu	n	CSA	n	14	d31-d145
2	A2, B35	MDS (pr)	f/m	32/36	+/+	m allo PBSC M UR	Flu, Mel	n	CSA, MMF	n	35	d88-d902
3	A1, B35	EP	m/m	63/32	+/-	m allo PBSC M UR	Cam, Flu, Mel	n	TA	n	30	d33-d512
4	B35	AML-M4	m/m	44/38	+/+	allo PBSC M sib	Cy, TBI	n	CSA, MTX	n	37	d46-d734
5	A1, B35	ALL-CR3	m/m	26/unknown	+/+	allo BM M sib	Eto, TBI	n	CSA, MTX	n	9	d53-d156
6	A2, B35	B-CLL	m/m	44/47	+/+	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	n	14	d23-d198
7	A24, B35	AML-M4, CR1	f/f	36/39	+/-	allo BM M sib	Cy, TBI	n	CSA, MTX	n	20	d46-d818
8	B35	AML (tr1)	f/f	55/43	+/+	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	n	4	d40-d60
9	B35	MM IgAκ	f	42	+	auto PBSC	Mel	n	-	n	12	d33-d808
10	A24	T-ALL	m/f	27/0	+/+	CB M UR + TPD MM sib	ATG, Cyt, Flu, TBI	n	CSA	n	11	d50-d360
11	A24	CML Ph+	f/f	57/28	+/+	m allo PBSC M UR	Cam, Flu, Mel	n	CSA	n	35	d99-d791
12	A24	AA	m/f	36/30	+/+	allo BM M sib	ATG, Cy	n	CSA	n	5	d54-d94
13	A2, A24	MM IgGκ	m	46	+	auto PBSC	Mel	n	-	n	9	d21-d774
14	A1, A24	MMλ	f	51	+	auto PBSC	Mel	n	-	n	6	d27-d194
15	A2, A24	CLL	f/f	51/43	+/-	m allo BM M sib	Cy, Flu	n	CSA, MTX	n	8	d11-d156
16	A24	HD (IVB)	m	20	+	auto PBSC	Eto, Mel	n	-	n	7	d36-d95
17	A24	MDS	m/m	41/30	+/+	allo BM M UR	Cy, Flu, TBI	y	CSA	n	7	d39-d118
18	A24	AML-CR2 (tr2)	f/f	58/43	+/+	m allo PBSC M UR	Cam, Flu, Mel	n	CSA, Def	n	22	d64-d830
19	A24	T-ALL-CR1	m/f	25/25	+/+	allo PBSC M sib	Cy, Flu	y	unknown	n	14	d56-d140
20	A24	CML Ph+	m/f	54/50	-/+	allo PBSC M UR	Cy, TBI	n	CSA, MMF, MTX	n	6	d32-d70
21	A1	FasL	m/f	32/28	+/-	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	n	33	d55-d682
22	A1	AML-CR1	f/m	44/41	+/+	allo PBSC M sib	Cy, TBI	CAM	CSA	n	18	d20-d384
23	A1, A2	MMκ-CR1	f/f	49/42	+/+	m allo PBSC M sib	Flu, TBI	n	CSA, TA	y	9	d38-d185
24	A1, A2	myelofibrosis	m/f	66/60	+/+	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	y	5	d32-d155
25	A1	ALL Ph+	m/m	19/33	+/-	allo PBSC MM UR	Cy, Flu, TBI	y	CSA	n	5	d90-d136
26	A1, A2	XLP	m/m	18/unknown	+/+	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	n	12	d33-d296
27	A1	AML-CR1 (tr3)	f/m	43/38	-/+	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	n	9	d83-d209
28	A1	HD (IVA)	m/m	21/32	-/+	m allo PBSC, MM UR	Cam, Flu, Mel	n	CSA	n	8	d64-d155
29	A2	B-CLL	m/f	53/42	+/+	m allo PBSC M sib	Cam, Flu, Mel	n	CSA	n	8	d52-d197
30	A2	NK-NHL	m/m	56/unknown	+/-	m allo PBSC MM UR	Cam, Flu, Mel	n	CSA	y	39	d33-d669

Continued

Continued

Patient ID	GvHD/systemic treatment	Outcome
1	unknown	unknown
2	a GvHD, severe skin and lung c GvHD / Prednisolone and MMF	3 years post Tx: in remission, on Cyclosporine for cGvHD
3	mild skin GvHD/ no treatment	2 years post Tx: cured, on steroids, numerous atypical infections as a result of immunosuppression
4	a GvHD untreated, severe chronic skin and lung GvHD / Prednisolone and MMF	3 years post Tx: several recurrent chest infections, on systemic steroids due to cGvHD
5	none	4 1/2 months post Tx: 3rd relapse, extremely poor prognosis
6	none	1 1/2 years post Tx: mixed chimaerism treated with DLI
7	skin a and c GvHD grade 1-2 / Prednisolone and MMF	2 years post Tx: recovered from GvHD, well
8	severe gut a GvHD grade 3 / MMF	Died at day 82 post Tx after severe steroid-refractory gut GvHD and sepsis (pneumonia)
9	none	3 1/2 years post Tx: relapse, considered for allo Tx
10	none	1 ¾ years post Tx: well, in remission
11	skin a and c GvHD / Prednisolone and MMF	3 1/2 years post Tx: in molecular remission, well
12	none	2 years post Tx: well, 85% donor chimaerism
13	none	2 1/2 years post Tx: recurrence of disease, no UR donor available for allo Tx
14	none	1 year post Tx: relapse
15	none	1 1/2 years post Tx: well, in remission
16	none	Day 95 post Tx: well
17	a GvHD, limited c GvHD (grade 2) / no systemic treatment	4 months post Tx: in remission
18	none	3 1/4 years post Tx: well, in remission
19	unknown	1 1/2 years post Tx: in remission
20	steroid refractory grade IV gut a GvHD / Prednisolone	Died at day 83 post Tx after severe steroid-refractory gut GvHD and hepatic encephalopathy
21	c GvHD of skin, eye, throat since day 328 post Tx,/ Prednisolone	3 years post Tx: full chimaerism, normal platelet count, no auto Ab, recovered from GvHD, well
22	skin a GvHD, grade 2 / Prednisolone	2 years post Tx: in remission
23	skin c GvHD / MMF, Prednisolone and Tacrolimus	2 years post Tx: in complete remission
24	none	Died at day 158 post Tx after recurrence of severe gut CMV disease
25	mild skin c GvHD / no systemic treatment	5 months post Tx: well
26	none	1 1/2 years post Tx: chimaerism 50:50, treatment with DLI
27	skin a GvHD grade 1 / Prednisolone and MMF	Day 193 post Tx: well
28	none	1 year post Tx: relapse
29	skin c GvHD / Prednisolone	1 year post Tx: in remission
30	skin a and c GvHD grade 2 / Prednisolone	3 years post Tx: well, in remission

Table 2-1 Characteristics of patients of the follow-up cohort

This table lists all patients included in the follow-up cohort and their characteristics.
Abbreviations: Ab: antibody; AA: aplastic anaemia; ALL: acute lymphoblastic leukaemia; allo: allogeneic transplant; AML: acute myeloid leukaemia (M4: stage of disease); ATG: anti-thymocyte globulin; auto: autologous transplant, B-: disease is associated with B cells; BM: bone marrow; Bus: Busulfan; Cam: Campath received *in vivo*; CAM: Campath in the bag; CLL: chronic lymphocytic leukaemia; CML: chronic myeloid leukaemia (Ph+ refers to Philadelphia chromosome positivity); CMV disease: cytomegalovirus disease with proven organ involvement (CMV PCR positive tissue biopsy); CR1-3: in first to third complete remission; CSA: Cyclosporine; Cy: Cyclophosphamide; Cyt: Cytarabine; Def: Defibrotide; DLI: donor lymphocyte infusion; EP: Erythropoietic protoporphyria; Eto: Etoposide; f; female; FasL: Fas Ligand deficiency; Flu: Fludarabine; (a, c) GvHD: (acute, chronic) graft *versus* host disease (listed are systemic treatments only); HD: Hodgkin's disease (IV A and IV B: stages of disease); HLA: human leukocyte antigen; HSC: haematopoietic stem cell; HSCT: haematopoietic stem cell transplantation; IgA k: immunoglobulin A kappa light chain; IgG k: immunoglobulin G kappa light chain; l: lambda light chain; M: HLA identical (matched); m: : mini-transplant involving reduced-intensity conditioning (HSC source) or male (sex); MDS: myelodysplastic syndrome; Mel: Melphalan; MM: multiple myeloma (disease) or mismatched/HLA non-identical (HSC source); MMF: mycophenolate mofetil; MTX: Methotrexate; n: no; NHL: Non-Hodgkin's Lymphoma; NK-: disease related to natural killer cells; PBSC: peripheral blood stem cell; pr: progressive; serol.: serology; sib: related (sibling) donor; T-: disease is associated with T cells; TA: Tacrolimus; TBI: total body irradiation; TCD: T cell depletion; tr1: related to therapy of breast cancer; tr2: transformed from MDS; tr3: related to therapy for previous HD; Tx: transplantation; UR: unrelated (donor); XLP: x-linked lymphoproliferative disease; y: yes

* disease: (CMV) disease is indicated by a tissue biopsy testing positive for CMV PCR
** no: the number of samples included in the follow-up analysis
*** period: indicates the period of follow up shown in days post transplantation (d)
**** Patient 10 received a dual transplant with donor information shown associated with the cord blood (CB) stem cells received, which were HLA matched at the HLA-A24 locus. Third party donor (TPD) stem cells were unmatched at the HLA-A24 locus.

Patient ID	HLA of interest	Diagnosis prior to HSCT	Sex (r/d)	Age (r/d)	CMV serol.(r/d)	HSC source	Conditioning	TCD of graft
30b	A2, B35	NK-NHL	m	55	+	auto BM	Cyt, Eto, Mel	none
31	A1, B35	MM	m	52	+	auto PBSC	Mel	none
32	A2, B35	NHL	f	43	+	auto PBSC	BEAM	none
33	A1	CML	m/f	32/22	+/-	allo BM	Cy, TBI	none
34	A1, A24	AML M4	f/m	30/39	+/+	allo PBSC	Cy, Flu, TBI	CAM
35	A2, B35	ß Thal	m/m	31/16	+/+	allo PBSC	Bus, Cam, Flu	none
36	A24	NHL	m	49	+	auto BM	CCNU, Cy, Cyt, Eto, Rituximab	none
37	A2	MM	m	60	+	auto PBSC	Mel	none
38	A2	MM	f	60	+	auto PBSC	Mel	none

Table 2-2 Characteristics of patients other than of the follow-up cohort

This table lists all patients and their characteristics for whom measurements are shown within this thesis but who were not eligible to be included in the follow-up cohort.
Abbreviations: allo: allogeneic transplant; AML: acute myeloid leukaemia (M4: stage of disease); auto: autologous transplant, BEAM: conditioning comprising Etoposide, BiCNu (carmustine) and Melphalan; ßThal: ß Thalassaemia; BM: bone marrow; Bus: Busulfan; Cam: Campath received *in vivo*; CAM: TCD by Campath in the bag; CCNU: lomustine, an alkylating agent related to BCNU (carmustine); Cy: Cyclophosphamide; Cyt: Cytarabine; Eto: Etoposide; f: female; Flu: Fludarabine; HLA: human leukocyte antigen; HSC: haematopoietic stem cell; HSCT: haematopoietic stem cell transplantation; ID: identification number; m: male; Mel: Melphalan; MM: multiple myeloma; NHL: Non-Hodgkin's Lymphoma; NK-: disease related to natural killer cells; PBSC: peripheral blood stem cell; serol.: serology; TBI: total body irradiation; TCD: T cell depletion.

Subject ID	HLA of interest	Sex	Age	CMV serology
1	A1, A2, B35	m	53	+
2	B35	f	50	+
3	A1, B35	f	40	+
4	B35	f	31	+
5	A2, B35	f	unknown	+
6	A2, B35	m	47	+
7	A24	m	36	+
8	A2, A24	f	52	+
9	A24	f	28	+
10	A2, A24	m	46	+
11	A24	f	58	+
12	A1	f	28	+
13	A1	m	43	+
14	A1	m	61	+
15	A2	f	34	+
16	A2	f	38	+
17	-	m	28	-
18	A2	f	27	-
19	-	f	23	unknown

Table 2-3 Brief characteristics of healthy blood donors

This table lists all healthy volunteers who donated blood for this study, and their characteristics.
Abbreviation: f: female; ID: identification number; m: male.

2-3 Processing of blood samples

50-100 µl of heparinised blood was used to obtain absolute T cell counts whereas the remainder of each sample was used for cell separation. Both methods are described below.

2-3.1 Absolute cell counts

Heparinised blood samples were used for determination of T cell counts per volume of blood. This analysis was performed at the day of sampling. Initially 2 tests each requiring 50 µl of heparinised blood were performed.

20 µl of TriTEST antibody mix (Becton Dickinson (BD) Biosciences) containing either fluorescein isothiocyanate (FITC) conjugated anti-CD3, phycoerythrin (PE) conjugated anti-CD4 and peridinin chlorophyll protein (PerCP) conjugated anti-CD45 or FITC conjugated anti-CD3, PE conjugated anti-CD8 and PerCP conjugated anti-CD45 were added to each of two TruCOUNT tubes, which contained a dry pellet of a known number of beads (BD Biosciences). The exact volume of 50 µl blood was then added to each of the TruCOUNT tubes and incubated for 15 minutes in the dark. After a further 15 minutes incubation with exactly 500 µl fluorescent-activated cell sorting (FACS) Lysing Solution (BD Biosciences), stained samples were immediately acquired on a FACSCalibur flow cytometer with four-colour fluorescence capability (BD Biosciences) using Multiset software (BD Biosciences) and calibration with CaliBRITE beads (BD Biosciences). Collaborators followed the exact same procedure after visiting the ANRI laboratories. A representative example of automatic cell gating by this software is shown in Figure 2-1.

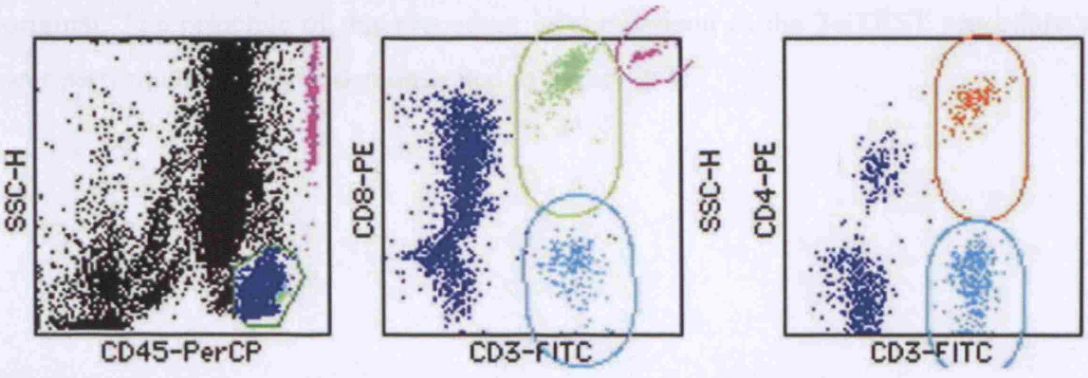


Figure 2-1 Gating of cell populations by Multiset software.

Flow cytometry plots represent automatic detection of beads (purple), $CD45^+$ $CD3^+$ $CD4^+$ and $CD45^+$ $CD3^+$ $CD8^+$ T cells by Multiset software for calculation of absolute T cell counts per volume of blood.

The use of 0.6x diluted antibody mix and usage of the MultiTEST antibody mix containing anti-CD45 PerCP, anti-CD3 FITC, anti-CD8 PE and anti-CD4 conjugated to Allophycocyanin (APC) were later verified in control experiments on ten test samples. This is demonstrated in Figure 2-2.

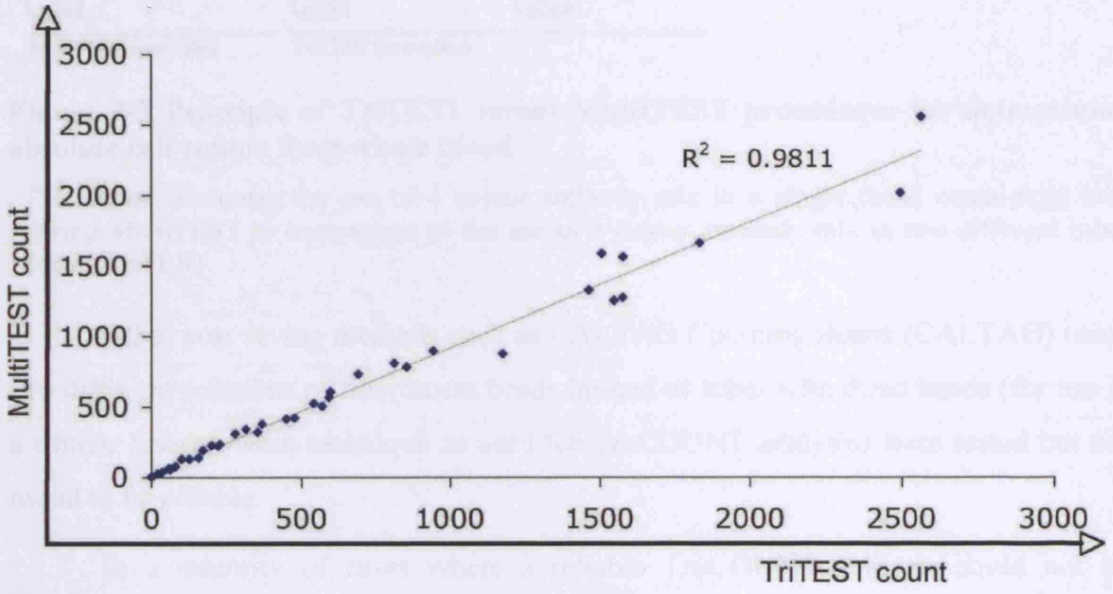


Figure 2-2 Correlation of TruCOUNT results obtained by staining with TriTEST or MultiTEST antibody mix

This figure demonstrates correlation between the results obtained by TruCOUNT analysis using staining with TriTEST and with MultiTEST antibody mix.

Subsequently absolute counts were obtained by staining 50 µl of heparinised blood with 20 µl of 0.6X diluted MultiTEST antibody in a single TruCOUNT tube. The alteration of the procedure enabled efficient use of financial resources by reducing the number of required TruCOUNT tubes by half and the amount of antibody mix to 30 % of the original. The principle of this procedure in comparison to the TriTEST procedure that was performed initially is demonstrated in Figure 2-3.

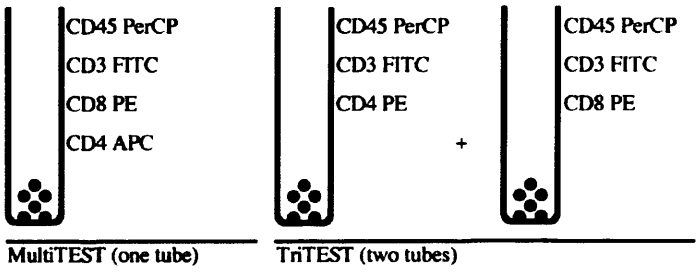


Figure 2-3 Principle of TriTEST *versus* MultiTEST procedures for determining absolute cell counts from whole blood

This figure illustrates the use of 4 colour antibody mix in a single (bead containing) tube during MultiTEST in comparison to the use of 3 colour antibody mix in two different tubes during TriTEST.

Other cost saving methods such as CALTAG Counting Beads (CALTAG) using two different solutions of fluorescent beads instead of tubes with dried beads (for use in a similar lyse-no-wash technique as used for TruCOUNT analysis) were tested but not found to be reliable.

In a minority of cases where a reliable TruCOUNT analysis could not be obtained from blood samples at the day of sampling, T cell counts per volume of blood were calculated by referring to lymphocyte counts obtained from routine analysis at the RFH and the percentage of $CD4^+$ and $CD8^+$ T cells of these lymphocytes obtained by flow cytometry staining. The preservative Cyto-Chex BCT (Streck Laboratories) was verified for use of TruCOUNT analysis in blood for up to 5 days after sampling for future studies (not shown).

2-3.2 Cell separation and storage

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation. This was performed by layering 10 ml blood over 10 ml Lymphoprep (Nycomed) and centrifugation at 2,200 rpm (no break) for 20 minutes. The Ficoll contained in Lymphoprep is a neutral high mass hydrophilic polysaccharide. Centrifugation of blood over this reagent results in separation of layers containing plasma and other constituents on the top, PBMC below, Ficoll below that and erythrocytes and granulocytes as a pellet on the bottom. PBMC were then harvested from the middle layer and washed twice in RPMI 1640 medium with L-Glutamine (BioWhittaker). Resuspension for subsequent use was performed in the same medium supplemented with 10 % heat inactivated (at 55 °C for 1 hour) fetal calf serum (FCS), 1 U/ml penicillin and 1 µg/ml streptomycin (both BioWhittaker). This supplemented medium will subsequently be referred to as complete medium.

2-4 Cell culture

2-4.1 Cell enumeration and viability

Cell counts were performed by dye exclusion method. Therefore 10 µl of the homogeneous cell suspension were stained with an equal amount of sterile filtered phosphate buffered saline (PBS) solution containing 0.4 % (w/v) trypan blue (BDH). Viable (non-stained) cells were counted in a haemocytometer (Neubaur Chamber, Weber) under a phase contrast Leica DM LB microscope (Meyer Instruments).

2-4.2 Cryopreservation of cells

Cells that needed to be stored for use at later time points were cryopreserved in pre-cooled freezing mix containing heat inactivated FCS and 10 % dimethyl sulfoxide (DMSO) (BDH). Therefore 1 ml aliquots containing a maximum of $1x10^7$ cells each were transferred to 1.5 ml cryotube vials (Nunc) and immediately placed in freezing containers (Sigma Aldrich) at -80 °C for 24 hours for an uniform cooling rate of 1 °C per minute from ambient temperature (by indirect immersion in an isopropyl alcohol bath) before storage in liquid nitrogen.

PBMC isolated from density gradient centrifugation layers with red blood cell trapping (that occurred in a minority of samples) were preferentially cryopreserved before further use because freezing resulted in reduction of contaminating red blood cells.

Cryopreserved samples were put back in culture by rapid warming at 37 °C, followed by at least two washing steps in 50 ml RPMI 1640 (Bio Whittaker) using centrifugation at 1,600 rpm for 10 minutes and incubation in complete medium for a few hours prior to further use to allow for recovery of cell surface molecules and cellular function.

2-4.3 General culture of cells

PBMC were usually incubated in complete medium (described in section 2-3.2). Hybridoma and EBV-transformed cell lines were cultured in the same medium in T-75 flasks (FALCON) at 37 °C/5 % CO_2 in a humidified incubator (IG 150, Jouan). All cell cultures were handled under semi-sterile conditions in a fume hood and tested for mycoplasma contamination using the VenorGeM Mycoplasma Detection Kit (Cambio Ltd) on a regular basis. This does not include cell culture of *Escherichia coli* strains, which is described in section 2-11.3. Half of the culture medium was typically

replenished every 2 to 3 days or when change in pH (and therefore colour of the cell culture medium) indicated medium exhaustion.

Mycoplasma pneumoniae lack a cell wall and are therefore resistant to the effects of penicillin (which acts by disrupting the bacterial cell wall) that was used in complete medium to prevent infection of cell cultures with bacteria. To screen cell cultures for contamination with *Mycoplasma* or *Acholeplasma*, 100 µl of supernatant from the test culture were boiled at 95 °C for 5 minutes. After brief centrifugation to pellet cellular debris, the supernatant was used for PCR detection with *Taq* (Bioline) according to the manufacturer's protocols.

The epithelial-like adherent choriocarcinoma cell line JAR (ATCC HTB-144) was grown under the conditions described above with half of the medium replenished with fresh medium every two to three days. Cells were passaged if they were greater than 80 % confluent by washing with sterile PBS followed by incubation with a premixed solution (GIBCO) containing trypsin and ethylene diamine tetraacetic acid (EDTA) at 37 °C for two minutes. Trypsin was removed by centrifugation and subsequent resuspension of cells in fresh complete medium.

The hTERT-BJ1 cell line (Clontech) was grown by Aycan Walker (Virology Department, Royal Free Hospital, London) in a 4:1 ratio of Dulbecco's Modified Eagle's Medium (DMEM, Sigma): Medium 199 (Sigma) with 10 % heat inactivated FCS (BioWhittaker), 1 mM sodium pyruvate (Invitrogen) and 4 mM L-glutamine (GIBCO). This cell line expressed exogenous human telomerase reverse transcriptase (hTERT) resulting in an indefinite life span. It was passaged in the same way as described for JAR above.

2-4.4 Production of antibodies by culture of hybridomas

Hybridomas producing W6/32 (murine IgG 2a monoclonal antibody reactive to fully assembled anti-HLA class I) (Barnstable *et al.*, 1978) or BB7.2 (IgG 2b monoclonal antibody reactive to HLA-A2) (Parham and Brodsky, 1981) were a kind gift from Dr. Walter Bodmer (Imperial Cancer Research Fund, ICRF, now called CRUK). They were cultured as described in section 2-4.3 and were used to harvest antibodies from their supernatant.

After centrifugation at 3,000 rpm for 10 minutes to pellet cellular debris, supernatant was stored at 4 °C before purification of secreted antibody by Affi-Gel Protein A Agarose kit (BIO-RAD), which uses the property of *Staphylococcus aureus*'s

Protein A that binds with high affinity to the F_c region of IgG (Kronvall and Williams, 1969). In brief, sample supernatant that had been passed through a microporous filter having a pore size of 0.4 μ, was passed through columns containing agarose beads coupled to protein A using a Peristaltic Pump P1 (Amersham Biosciences). Columns were washed and specifically bound antibody eluted at low pH with buffers supplied with the Affi-Gel Protein A Agarose kit (BIO-RAD).

2-4.5 CMV infection of cells

H-TERT-BJ1, JAR or other cells were plated at $8x10^5$ cells/dish in 5 different 35 mm Petri dishes. Infection experiments were performed in collaboration with Dr. Aycan Walker at the Virology Department at the RFH. Incubation at 37 °C/5 % CO_2 for half a day ensured that cells were allowed to attach. One dish was used as a negative control and left untreated. The medium from the remainder was aspirated and 2 ml enhanced green fluorescent protein (EGFP) recombinant viral supernatant of either TOLEDO-F (a fibroblast adapted TOLEDO strain) or AD169 supernatant added in duplicates. Both viral strains were a kind gift from Gavin Wilkinson, University of Wales College of Medicine. They had been grown in the hTERT BJ1 cell line and used at an MOI of approximately 1.

After incubation at 37 °C/5 % CO_2 for 90 minutes, the supernatant was aspirated and replenished with 2 ml of the medium originally used for culturing the cells. Cells were grown for 48 to 72 hours before GFP expression was assessed using a fluorescent microscope (Olympus B x60). Pictures were taken using an attached camera (Olympus DP11).

2-5 Fluorescent cell surface staining and analysis

2-5.1 Tetramer staining

The staining specificity of tetramers is temperature dependent with an optimum specificity obtained at 37 °C (Whelan *et al.*, 1999). Whelan and colleagues demonstrated that peptides that are unable to elicit functional $CD8^+$ T cell responses, can stain these cells at 4 °C but not 37 °C when incorporated into tetramers. They therefore suggest that staining at 4 °C can detect cross reactive $CD8^+$ T cells with low avidity for the tetramer more readily than staining at 37 °C. Therefore all tetramer-staining procedures were performed at this temperature.

PBMC were stained in phosphate buffered saline (PBS) diluted from a 10x stock solution (Sigma-Aldrich) supplemented with 0.5 % FCS and 0.1 % (w/v) sodium azide (NaN_3) (BDH) with the appropriate volume of tetramer solution at 37 °C for 30 minutes. Tetramer solutions were titrated to establish the volume required for optimum staining results. Amounts added were typically 1µg of fluorescent tetramer solution in a staining volume of 50 µl. Control staining was performed using PBMC from individuals expressing irrelevant HLA alleles and CMV seronegative individuals.

After centrifugation of tetramer stained cells at 1,600 rpm for 3 minutes and one washing step in staining buffer, cells were resuspended in 50 µl PBS/FCS/NaN_3 and stained with 3 µl of FITC labelled anti-CD3 and PerCP labelled anti-CD8 antibodies (Becton Dickinson) at 4 °C for 20 minutes.

In some cases the second staining step included anti-CD3 or anti-CD8 antibody staining with fluorochrome combinations other than those mentioned above to allow for staining with a fourth reagent using a combination of labels including FITC, PE, PerCP and APC.

Washing thereafter was performed twice with PBS/0.1 % (w/v) NaN_3. Cells were then fixed in PBS/1 % (w/v) paraformaldehyde (PFA) (BDH) to ensure that bound antibodies and tetramer did not dissociate from their ligand molecule on the cell surface, and were stored at 4 °C in the dark. Acquisition on a FACSCalibur flow cytometer (Becton Dickinson) was performed within 24 hours using CellQuest version 3.3 software (Becton Dickinson) and analysed using FlowJo software Version 6.0 (Tristar).

2-5.2 Staining with other reagents

Cell surface staining not involving tetramers was performed in PBS supplemented with 0.5 % FCS at 4 °C for 20 minutes followed twice by centrifugation at 1,600 rpm for 3 minutes and washing in staining buffer. Cells were then fixed in PBS/1 % (w/v) PFA and stored at 4 °C in the dark until acquisition within 24 hours.

Many immunologically important cells can be defined based on what molecules are present on their surface. Numbered human clusters of differentiation (CD) are used to classify many epitopes on the cell surface of leukocytes. A combination of several surface markers is often used to associate cells with certain immune functions or properties.

Antibodies directed against different CDs were purchased from BD Pharmingen. These had fluorochromes FITC, PE, PerCP or APC directly conjugated and were used

as described above. Antibodies used had the following epitope specificities (clones in brackets) and fluorochromes: CD3 (HIT3a) FITC, CD3 (SK7) PerCP, CD3 (SK7) APC, CD4 (RPA-T4) FITC, CD4 (SK3) PE, CD4 (SK3) PerCP, CD8 (SK1) PE, CD8 (SK1) PerCP, CD16 (NKP15) FITC, CD19 (4G7) FITC, CD25 (2A3) PE, CD27 (M-T271) FITC, CD28 (L293) PE, CD45RO (UCHL-1) PE, CD56 (B159) PE, CD57 (HNK-1) FITC, CD69 (L78) FITC.

Other antibodies used included anti-Vß 7 antibody (clone ZOE, Immunotech) and anti-Vß 20 antibody (clone ELL1.4, Immunotech). These antibodies were not directly conjugated to fluorochromes. Therefore detection required staining with a secondary antibody. Primary antibodies were composed of mouse IgG heavy chain. Therefore FITC conjugated sheep anti-mouse IgG (whole molecule) antibody (Sigma Aldrich) was used as the secondary detection antibody. Briefly, cells were stained with 2 µl anti-Vß7 or 4 µl anti-Vß20 antibody (volumes based on titration results) in PBS/FCS/NaN_3 in a total staining volume of 100 µl at 4 °C for 20 minutes followed twice by centrifugation at 1,600 rpm for 3 minutes and washing with staining buffer. Cells were then stained with 4 µl of FITC conjugated sheep anti- mouse IgG antibody in a total volume of 100 µl staining buffer at 4 °C for 30 minutes followed twice by centrifugation at 1600 rpm for 3 minutes and washing in staining buffer. Cells were then fixed in PBS/1 % (w/v) PFA and stored at 4 °C in the dark until acquisition within the next 24 hours.

HLA specific staining during preliminary experiments (not shown) was performed with the HLA-A24 specific murine IgG antibody 030041HA (One Lambda), the HLA-B35 specific human IgG antibody HDG8D and the HLA-A1/24 specific human IgG antibody GV5D1. The latter two were obtained from Dr. Arend Mulder (Leiden University Medical Centre) and were used with FITC conjugated rabbit $F(ab)_2$ anti-human IgG secondary antibody (DakoCytomation).

Staining with non-fluorescent antibodies such as biotinylated antibodies or those conjugated to peroxidase is described in the sections describing the relevant assays (for example dot blot, western blot, ELISpot and cell selection). Staining with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDASE) or intracellular cytokine (ICC) staining is described in sections 2-7.3 and 2-7.2 respectively.

2-5.3 Flow Cytometry

Cells stained with fluorochrome-conjugated antibodies were visualised using flow cytometry. This technique is based on the principle that a beam of a single wavelength light is directed onto a stream of fluid. Detectors are aimed at the point where the stream passes through the light beam. In this study a FACSCalibur flow cytometer (Becton Dickinson) was used. This machine has one detector positioned in line with the light beam, called forward scatter (FSC), and five detectors positioned perpendicular to it. The last mentioned are the side scatter (SSC) and four fluorescent detectors. These detect green FITC emission (maximum 530 nm), orange PE emission (maximum 585 nm) or red PerCP emission (maximum 650 nm), all excited by a blue argon laser (488 nm); or long red-blue APC emission (maximum 670 nm) excited by a red diode laser (635 nm). Each cell within the acquired liquid that passes through the beam scatters light. Light scattered in forward direction is directly proportional to the size of the cell (FSC) whereas light scatter at larger angles to the side (SSC) is caused by granularity and structural complexity of the cell. In addition fluorochromes on that cell are excited into emitting light. Light scattered to the sides is focused through a lens system and is collected by the SSC detector located at around 90 degrees from the laser's path. Fluorescent light travels along the same path as the side scatter signal and is detected through a series of filters and mirrors so that particular wavelength information is delivered to the appropriate detector. The combination of scattered and fluorescent light that is picked up by the detectors is eventually converted to electronic signals.

Filters are used in order to separate fluorescence emission from the excitation light source and to resolve different colours. Detection channels (FL) therefore pass light in a specified range of wavelength (band-pass). Fluorochromes, however, emit light over a range of wavelengths and a signal from one fluorochrome may therefore overlap in a detector used for another fluorochrome. This is demonstrated in Figure 2-4.

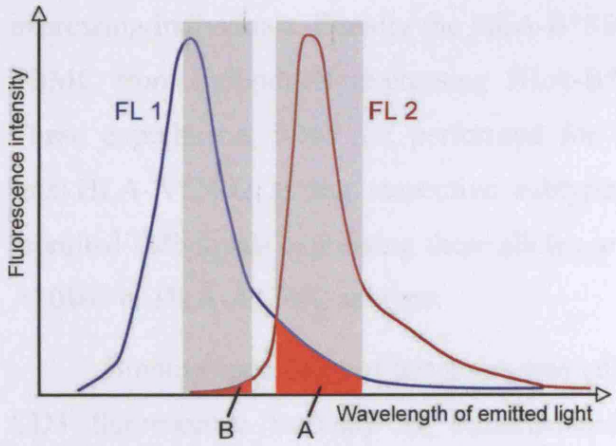


Figure 2-4 Spectral overlap

This figure represents the emission spectrum of FITC (FL1) in blue and the emission spectrum of PE (FL2) in dark red. Some FITC fluorescence detected within the band-pass (grey shaded) of the FL1 detector also appears within in the FL2 detector (A) and *vice versa* (B). This spectral overlap is represented in bright red.

Spectral overlap was manually compensated using control samples, each stained with a single colour in CellQuest software Version 3.3 (Becton Dickinson). Thereby a portion of one detector's signal was subtracted from another, leaving only the desired signal.

For analysis of patient samples, 20,000 $CD3^+$ $CD8^+$ live lymphocyte events were collected whenever possible. The number of CMV specific $CD8^+$ T cells per microlitre of blood was determined by multiplying the absolute number of $CD8^+$ T cells determined by TruCOUNT analysis (compare section 2-3.1) by the percentage of $CD8^+$ T cells, which bound to tetramer and fell within the live $CD3^+$ lymphocyte gate as determined using flow cytometry (Figure 2-6, page 112). The results were considered positive if a distinctive population was seen for $CD8^+$ T cells but not $CD8^-$ T cells.

Specificity of tetrameric complexes was confirmed by the absence of staining of cells from CMV seronegative donors expressing the appropriate HLA tissue type and the absence of staining of cells from CMV seropositive donors not expressing the appropriate HLA molecule. Only tetramers staining positive controls (PBMC from CMV seropositive individuals expressing the relevant HLA allele who had shown a response to the relevant peptide in ELISpot assays) without staining of negative controls were used after the volume required for optimum staining results had been establish in titration experiments.

Further experiments confirming the staining specificity revealed no cross-reactivity of HLA-A*0201/NLV tetramer in HLA-A*0205 and HLA-A*0217

expressing individuals. Equally the HLA-B*3501/IPS tetramer did not cross-react with PBMC from individuals expressing HLA-B*3503, HLA-B*3508 or HLA-B*3502. These experiments were not performed for tetramers comprising HLA-A*0101 or (ms) HLA-A*2402 as the respective subtypes were so predominant that typing of recruited individuals expressing these alleles revealed an exclusive expression of HLA-A*0101 or HLA-A*2402 subtype.

Binding specificity of tetramers was additionally confirmed by measurement of CD3 fluorescence intensity as demonstrated in Figure 2-5. Specific binding of tetrameric complexes to α and ß chains of the T cell receptor molecule influences the CD3 molecule due to its proximity. This has been shown to result in decreased staining with anti-CD3 antibody (Hoffmann *et al.*, 2000), whereas non-specific staining would not influence the binding of anti-CD3 antibody.

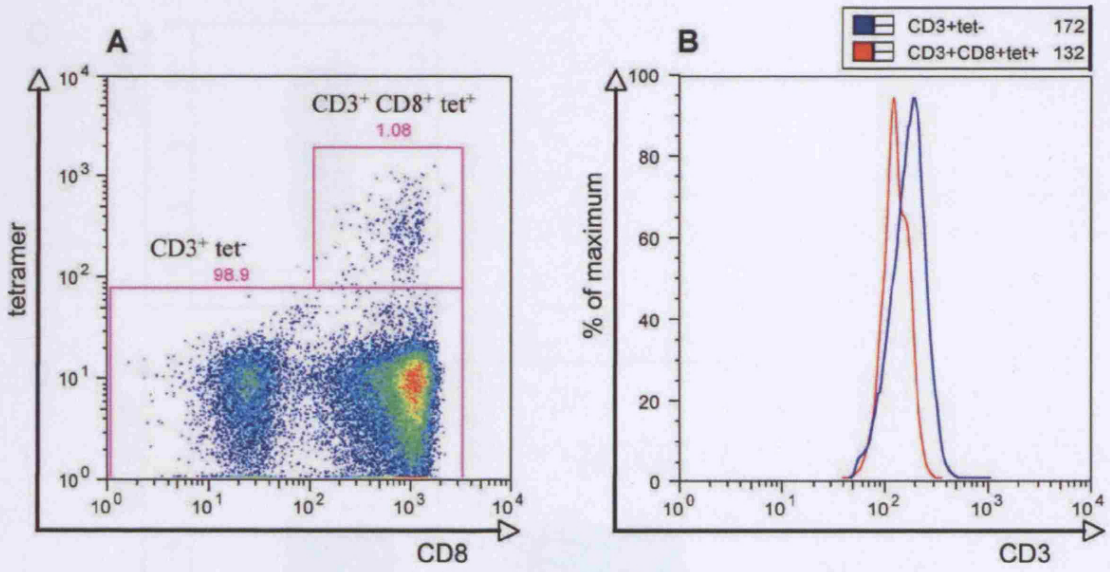


Figure 2-5 CD3 down regulation after tetramer staining

Part B of this figure demonstrates decreased staining of tetramer binding cells (red curve) with anti-CD3 antibody in comparison to cells that did not bind tetramer (blue curve). The gating of cell populations is shown in part A. The latter shows live $CD3^+$ T cells obtained from patient 12 at 54 days post HSCT, that were stained with HLA-A24/pp65(341-349) tetramer and anti-CD8 antibody as a representative example. Additionally to the graphical demonstration the geometrical mean of CD3-FITC fluorescence intensity is shown in the legend of part B.

Gating of tetramer stained PBMC was performed as illustrated in Figure 2-6 using FlowJo software Version 6 (Tristar).

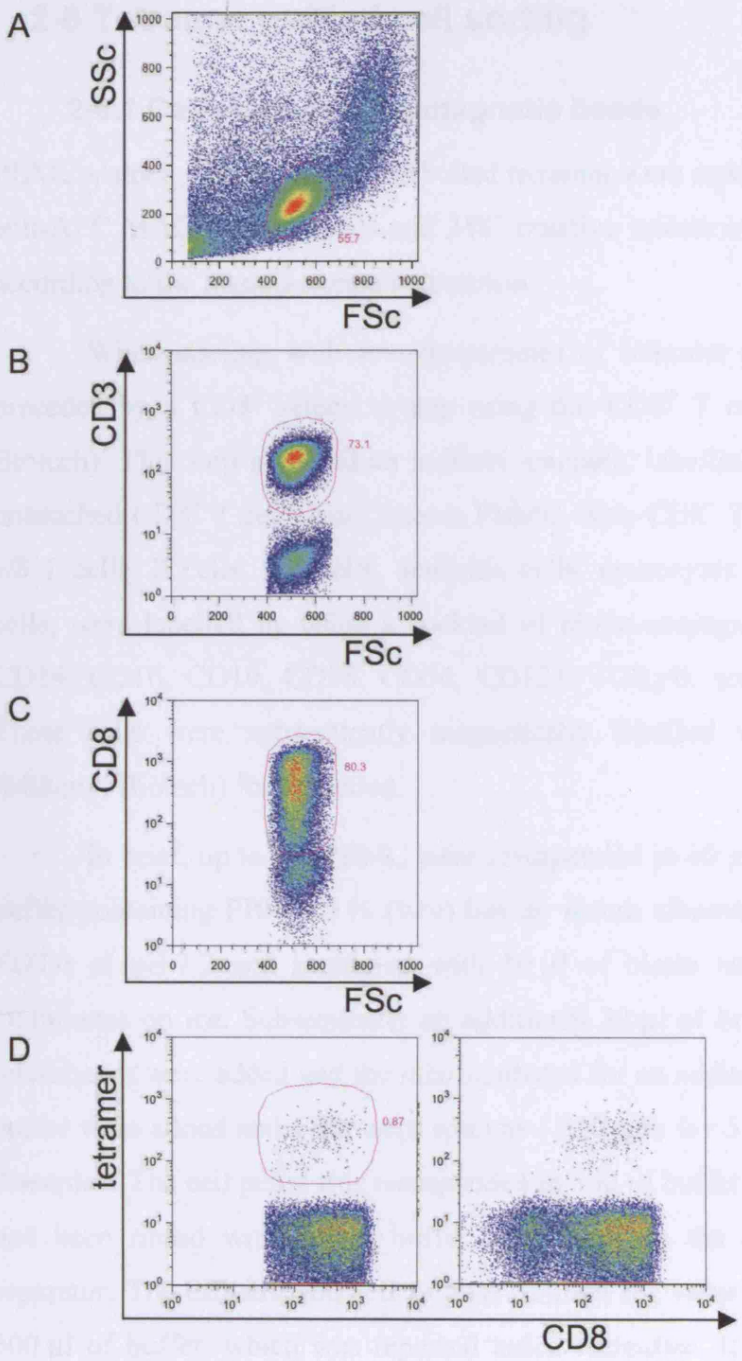


Figure 2-6 Boolean gating strategy used for tetramer FACS analysis

This figure demonstrates stepwise gating of PBMC from patient 12 stained with anti-CD3 antibody, anti-CD8 antibody and tetramer as a representative example. Part A illustrates total PBMC, of which live lymphocytes were gated according to their characteristic forward/side scatter (FSC/SSC) characteristics. Cells that fall within this first gate are shown in a new dot blot in part B. This was used to gate on the $CD3^+$ T cell population. Part C shows cells that fall within both of the first two gates. A third gate was drawn around the $CD8^+$ T cell population and cells falling within all three gates are shown in part D on the left. Those cells were analysed for binding with tetramer (here 0.87 %). Cells falling within the first two gates ($CD3^+$ T cells) are shown in part D on the right. This window demonstrates specific tetramer staining within the $CD8^+$ but not $CD8^-$ T cell population.

2-6 Tetramer guided cell sorting

2-6.1 Cell sorting using magnetic beads

PBMC stained with PE or APC labelled tetramer were separated on ice using anti-PE or anti-APC MACS micro beads and MS^+ positive selection columns (Miltenyi Biotech) according to the manufacturers instruction.

When starting with low frequencies of tetramer binding cells, selection was preceded by a $CD8^+$ selection step using the $CD8^+$ T cell isolation kit II (Miltenyi Biotech). This step involved an indirect magnetic labelling system for the isolation of untouched $CD8^+$ T cells from human PBMC. Non-$CD8^+$ T cells, such as $CD4^+$ T cells, γ/δ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells, were labelled by using a cocktail of biotin-conjugated antibodies against CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and CD235a (glycophorin A). These cells were subsequently magnetically labelled with anti-biotin microbeads (Miltenyi Biotech) for depletion.

In brief, up to 10^7 PBMC were resuspended in 40 µl of pre-cooled and degassed buffer containing PBS, 0.5 % (w/v) bovine serum albumin (BSA) (Sigma) and 2 mM EDTA at pH 7.2 and incubated with 10 µl of biotin labelled antibody cocktail for 10 minutes on ice. Subsequently an additional 30 µl of buffer and 20 µl of anti-biotin microbeads were added and the mix incubated for an additional 15 minutes on ice. 2 ml buffer were added and cells were spun at 1,500 rpm for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 500 µl buffer and applied to a column that had been rinsed with 500 µl buffer and placed in the magnetic field of a MACS separator. The effluent was left to pass through the column completely before adding 500 µl of buffer, which was repeated twice thereafter. It was collected as unlabelled $CD8^+$ T cell fraction and used for the second purification step.

PBMC were stained with PE or APC labelled tetramer as described in section 2-5.1 but using PBS supplemented with 0.5 % (w/v) BSA (Sigma) and 2 mM EDTA as staining buffer. 2 ml buffer were added and cells spun at 1,300 rpm for 5 minutes. The supernatant was removed and cells washed again before resuspension in 80 µl buffer and incubation with 20 µl anti-PE microbeads for 15 minutes on ice. Cells were then washed as before and added to a column prepared as described. After passing of 500 µl buffer through the column for three times, it was removed from the magnet, placed on a

collection tube and flushed with 1 ml buffer using the provided plunger to collect tetramer-PE labelled cells, which were passed over a second column to increase purity.

Sorting of PBMC was optimised by FACS sorting (refer to section 4-2.1.1), which is described in section 2-6.2.

2-6.2 Cell sorting by flow cytometry

Flow cytometry based sorting of tetramer binding cells for TCR Vß spectratyping (section 2-12) based on staining and gating of cells as described in section 2-5 was performed by Niga Nawroly at the flow cytometry laboratory, Department of Respiratory Medicine, Imperial College, London, UK using a FACS Aria instrument (Becton Dickinson).

2-7 Functional assays

2-7.1 Enzyme-linked immunospot (ELISpot)

Interferon gamma (INFγ) release by PBMC was detected with antibodies from the enzyme-linked immunospot (ELISpot) for human IFNγ kit (Mabtech) according to the manufacturer's protocol.

Briefly, 96 well MultiScreen 0.45 µm Immobilon-P (PVDF) filter plates (MAIPS4510, Millipore) were coated with the monoclonal anti-human IFNγ capture antibody (1-D1k) at 15 µg/ml and incubated at 4 °C overnight. Unbound antibody was washed off with 0.45 µm sterile filtered PBS (AccuGene). 10^5 PBMC in 200 µl complete medium were added to each well. All samples were tested in triplicates (a positive control was included in a single well). They were either incubated in the presence of the peptide of interest (10 µg/ml), PHA (2 µg/ml) as positive control or medium alone to test for spontaneous cytokine release. Cell-free medium was used as a negative control.

Plates were incubated at 37 °C/5 % CO_2 for 14 hours. Cells were then washed off with PBS for 6 times and plates incubated with 1 µg/ml biotinylated anti- human IFNγ detection antibody (7-B6-1-biotin) in PBS containing 0.5 % FCS for 2.5 hours at room temperature. Plates were washed 6 times with PBS again and incubated with a 1:1,000 dilution of streptavidin-alkaline phosphatase (provided with the kit) for 1.5 hours. Washed plates (6x with PBS) were finally incubated with 100 µl per well of alkaline phosphate substrate (0.1 M NaCl, 0.05 M $MgCl_2$ and 0.1 M Tris - HCl

(whereby Tris is used as abbreviation for trishydroxymethylaminomethane) at pH 9.5 containing 32 µl 5-bromo-4-chloro-3-indolylphosphate p-toluidine (BCIP, Gibco BRL) and 44 µl nitroblue tetrazolium chloride (NBT, Gibco BRL) per 10 ml buffer).

Colour development was usually observed after 2 minutes and stopped by washing plates with tap water. Plates were air dried and read on an automated counter. This was initially performed on an ELISpot AID System at the Immunology Department at the Hammersmith Hospital. When an ELISpot reader became available at Immunology Department of the RFH, all plates (including those initially read on the AID reader) were read using the same detection conditions using an Axiocam MR camera (Carl Zeiss) and SCHOTT KL 1500 LCD Cold Light source attached to a Stemi 2000-C Stereo Microscope (Carl Zeiss) with TANGO microscope controller (Märzhäuser) for analysis with KS ELISpot software Version 4.5.21 (Carl Zeiss). A representative example of results obtained from ELISpot assays is shown in Figure 2-7.

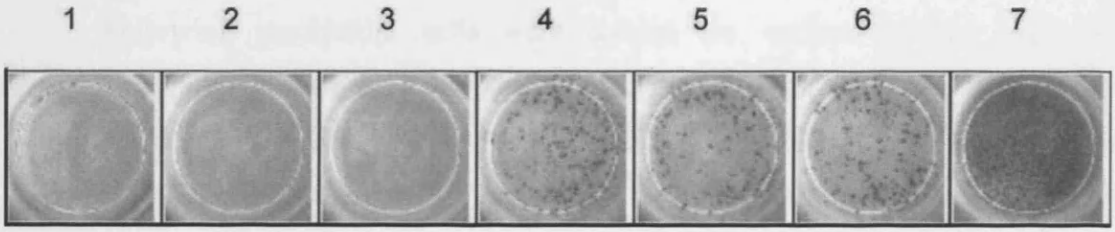


Figure 2-7 IFNγ ELISpot read out

This figure shows a typical result obtained from anti-human-IFNγ antibody coated wells in which 100,000 PBMC had been incubated in complete medium without stimulation (lane 1-3), with peptide (lane 4-5) or with PHA (lane 7) followed by removal of cells and detection of released IFNγ cytokine with biotinylated secondary antibody and streptavidin-alkaline phosphatase, which was visualised by addition of enzyme substrate resulting in coloured spots for each responding cell.

Samples showing no response to the positive control PHA were excluded from analysis. The median values of spots per 100,000 PBMC were calculated and deviations from triplicate measurements represented as 25 and 75 percentiles.

2-7.2 Intracellular cytokine (ICC) staining

For this procedure triplicates of $1x10^6$ PBMC each were incubated with or without peptide to establish IFNγ production. Staining with an isotype control was performed in addition to that. Due to the large number of cells required for this staining procedure ($9x10^6$ PBMC in total), functional assays were preferentially performed using ELISpot (compare section 2-7.1). In some cases confirmatory experiments were performed using intracellular staining (ICS).

Like ELISpot, ICS is a method for analysing cytokine production by single cells. The sensitivity of the method is lower (Tassignon *et al.*, 2005) but its advantage is the possibility to distinguish between subpopulations of cytokine producing cells by fluorescence-activated cell sorting (FACS) analysis without the need for prior isolation of the subpopulations.

Cells were resuspended in 500 µl RPMI (BioWhittaker) supplemented with 10 % heat inactivated human AB serum (CAMBREX), 1 U/ml penicillin and 1 µg/ml streptomycin (both BioWhittaker), 10 ng/ml Brefeldin A (BFA) (Sigma), 1 µg/ml anti-CD28 antibody (BD), with or without 10 ng/ml CMV peptide (Table 2-5, page 129) for incubation at 37 °C/5 % CO_2 for 12 hours. BFA is a lactone antibiotic produced by fungi such as *Eupenicillium brefeldianum*. During the stimulation process it acts to inhibit transport to the Golgi complex thereby preventing the secretion of produced cytokines and resulting in their accumulation within the ER of cells instead.

Following incubation, cells were stained for surface marker expression. Therefore cells were spun at 1,600 rpm for 3 minutes, washed twice by resuspension in 100 µl PBS/1 % FCS followed by centrifugation at 1,600 rpm for 3 minutes and discarding of the supernatant. The viability of cells was then briefly checked by dye exclusion method (compare section 2-3.2). Cells were stained with 3 µl PE conjugated anti-CD3 antibody and 3 µl PerCP conjugated anti-CD8 antibody (BD) in 50 µl PBS/FCS at 4 °C for 30 minutes.

Following surface staining and two further washes, cells were fixed and permeabilised to allow entry of anti-cytokine antibodies into the cells which can then be determined by FACS analysis. Permeabilisation was achieved by incubation in 100 µl Cytofix/Cytosperm buffer (BD) at 4 °C for 20 minutes. Samples were subsequently washed in 100 µl Perm/Wash solution (BD) before staining with 2 µl of either FITC conjugated anti-human IFNγ antibody or FITC conjugated anti-mouse IFNγ antibody as isotype control in 50 µl Perm/Wash buffer at 4 °C for 30 minutes. Following two further washing steps in Perm/Wash, the samples were resuspended in PBS/1 % (w/v) PFA and stored at 4 °C in the dark for up to 24 hours before acquisition on a FACSCalibur flow cytometer (Becton Dickinson) (section 2-5.3).

2-7.3 Staining with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDASE)

PBMC were stained at 10^7 cells/ml in complete medium with 10 µM CFDASE from a 5 mM stock solution prepared in dimethylsulfoxide (DMSO) (Molecular Probes) at 37 °C in the dark for 10 minutes.

During the initial staining CFDASE passively diffuses into the cells, where two acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester (CFSE). The succinimidyl ester group reacts with intracellular amines to form fluorescent conjugates. A proportion of CFSE becomes coupled to long-lived intracellular molecules to form conjugates that cannot escape from the cells whereby stable fluorescent labelling is achieved (Parish, 1999).

CFSE stained samples were washed three times by resuspension in ice-cold complete medium followed by centrifugation at 1,600 rpm for 3 minutes and discarding of the supernatant. Cells were then incubated at $2x10^6$ cells/ml in complete medium at 37 °C in the dark for subsequent stimulation. Each stimulation was performed in triplicate. Therefore cells were either left unstimulated or stimulated with CMV peptide (10 µg/ml) or phytohaemagglutinin (PHA) (2 µg/ml) for a defined period of time. All samples were left in culture for the same length of time after staining with CFSE.

After culture cells were spun at 1,600 rpm for 3 minutes and washed twice in PBS/0.5 % FCS. They were resuspended in 50 µl PBS/FCS and stained with 3 µl of APC labelled anti-CD3 and PerCP labelled anti-CD8 antibodies (BD) at 4 °C for 20 minutes. Washing thereafter was performed twice with PBS. Cells were then immediately acquired on a FACSCalibur flow cytometer (BD) (section 2-5.3).

The dye-protein adducts that form in labelled cells are retained by cells throughout development and meiosis. The fluorescence intensity is progressively halved with each cell division and the amount of CFSE detected can thereby determine the degree of proliferation of cells since the time of staining.

2-8 General Molecular Biology applications

2-8.1 Plasmid DNA midiprep

This method was modified from the original alkaline lysis described by Birnboim and Doly in 1979 (Birnboim and Doly, 1979). Therefore 50 ml overnight cultures were spun at 3,000 rpm for 20 minutes and cell pellets resuspended in 1 ml of a solution containing 25 mM Tris and 10 mM EDTA at pH 8. Then 2 ml of freshly prepared solution containing 200 mM sodium hydroxide (NaOH) and 1 % (v/v) sodium dodecyl sulphate (SDS) was added and incubated on ice for 5 minutes. The lysis of bacteria under alkaline conditions results in precipitation of DNA and proteins. 3 M sodium acetate (acetic acid) at pH 4.8 was added and incubated on ice for 45 minutes. The acetate-containing buffer neutralizes NaOH in the previous lysis step. It results in large and less supercoiled chromosomal DNA (and proteins) forming an insoluble white, rubbery precipitate with SDS while small bacterial DNA plasmids can renature and stay in solution. After centrifugation at 4,000 rpm for 20 minutes, supernatant was added to tubes containing 8 ml of absolute ethanol, gently mixed by inversion and spun at 4,000 rpm at 4 °C for 10 minutes. 400 µl of solution containing 10 mM Tris and 1 mM EDTA at pH 8 was added to DNA pellets for incubation at 65 °C for 10 minutes. After addition of 500 µl lithium chloride (LiCl) the resulting solution was cooled to -20 °C for at least 10 minutes. It was then spun at 10,000 rpm at 4 °C for 10 minutes and 1 ml of absolute ethanol was subsequently added to supernatant resulting in precipitation of DNA. Further centrifugation at 10,000 rpm at 4 °C for 10 minutes was performed to enable aspiration of supernatant. The resulting DNA pellet was dried in a 37 °C incubator for 10 to 20 minutes before resuspension in 50 µl water and incubation in a water bath at 65 °C for 10 minutes.

2-8.2 Plasmid DNA miniprep

The midiprep method described above was the preferred method of plasmid DNA isolation because of high DNA yields with good purity. Occasionally a miniprep method was used when a short processing time was prioritised to high DNA yields.

DNA was extracted by StrataPrep plasmid miniprep kit (Stratagene) according to the manufacturer. This method employs a modification of the alkaline method of cell lysis (Birnboim and Doly, 1979) and binding of DNA on a silica-based fiber matrix.

Briefly 1.5 ml of a 10 ml overnight culture were spun at 13,000 rpm for 1 minute and the supernatant discarded. The pellet was resuspended in 100 µl of a ribonuclease-containing solution (50 mM Tris HCl (pH 7.5), 10 mM EDTA, 50 µg/ml RNase A) and gently mixed with 100 µl of a cell lysis solution (0.2 M NaOH, 1 % (w/v) SDS), and 125 µl of a DNA-binding salt solution (containing an undisclosed chaotropic salt). As described above, the lysis of bacteria under alkaline conditions results in precipitation of DNA and proteins. A salt neutralising buffer results in large and less supercoiled chromosomal DNA forming an insoluble white, rubbery precipitate with SDS while small bacterial DNA plasmids can renature and stay in solution. The sample was then spun at 13,000 rpm for 5 minutes. The DNA containing supernatant was then transferred to a cup with fibre matrix, to which the DNA binds. The cup was seated inside a receptacle tube and contaminants were subsequently washed from the cup with wash buffer. Therefore the tube was spun at 13,000 rpm for 30 seconds. After removal of liquid from the tube, 750 µl of wash buffer (5 mM Tris HCl (pH 7.5), 50 mM NaCl, 1.25 mM EDTA and 50 % (v/v) ethanol) were added to the cup and spun inside the tube at 13,000 rpm for a further 30 seconds. After removal of filtered liquid, the tube was spun for an additional 30 seconds and the cup was then transferred to a new tube. 50 µl sterile deionised water was placed on top of the fiber matrix and incubated at room temperature for 5 minutes for elution of the purified plasmid DNA, which was captured in the tube by centrifugation at 13,000 rpm for 30 seconds.

2-8.3 Absorbance measurement of DNA and RNA

DNA and RNA absorb ultraviolet light with an absorption peak at 260 nm wavelength. The amount of light absorbed can be related to the concentration of the absorbing molecule. At a wavelength of 260 nm, the extinction coefficient for double-stranded DNA is 20 $(mg/ml)^{-1}$ cm^{-1}; for single-stranded DNA and RNA it is 25 $(mg/ml)^{-1}$ cm^{-1}. Thus, an optical density (OD) of 1 corresponds to 50 µg/ml for double-stranded DNA, and to 40 µg/ml for single-stranded DNA and RNA.

Nucleic acid concentration was measured at 260 nm and protein contamination was assessed by the ratio of absorbance at 260 nm: 280 nm (which corresponds to 2.0 in pure nucleic acid solutions) using a spectrophotometer (Nanodrop).

2-8.4 DNA endonuclease restriction digest

Restriction endonucleases are enzymes that recognise specific sequences of nucleotides and produce a double stranded cut in the DNA. Two incisions, one through each of the sugar-phosphate backbones of the double helix are made without damaging nitrogenous bases. These enzymes have been named after discovery in *E. coli* strains that appeared to be restricting the infection by certain bacteriophages and are therefore believed to be a mechanism evolved by bacteria to resist viral attack. Recognition sites of restriction enzymes mentioned throughout this thesis are summarised in Table 2-4.

Enzyme	Source of Bacteria	Recognition site	Cleavage site
BamH I	*Bacillus*	GGATCC	G/GATCC
	amyloliquefaciens	CCTAGG	CCTAG/G
EcoR I	*Escherichia coli*	GAATTC	G/AATTC
		CTTAAG	CTTAA/G
Hind III	*Haemophilus*	AAGCTT	A/AGCTT
	influenzae	TTCGAA	TTCGA/A
Nco I	*Gordonia*	CCATGG	C/CATGG
	rubripertincta	GGTACC	GGTAC/C
Pst I	*Providencia stuartii*	CTGCAG	CTGCA/G
		GACGTC	G/ACGTC
Sal I	*Streptomyces albue*	GTCGAC	G/TCGAC
		CAGCTG	CAGCT/G

Table 2-4 Target sequences of restriction endonucleases

This table lists endonucleases that were used, their source, recognition sites and cleavage sites.

2-8.5 Gel electrophoresis

Gel electrophoresis is used for the separation of deoxyribonucleic acid or ribonucleic acid using an electric current applied to a gel matrix. Nucleic acids migrate from negative to positive electrodes due to the naturally occurring negative charge carried by their sugar-phosphate backbone. Gel electrophoresis can also be applied for proteins as is described in section 2-11.8 and 2-11.13.

Gels were prepared by heating 100 ml of 0.5x Tris-Borate-EDTA (TBE) (Cambrex Bio Sciences) buffer containing 1 g (unless stated otherwise) of electrophoresis grade agarose (Gibco BRL) in a microwave oven for approximately 2 minutes (until the agarose solution began to boil). The mix was cooled down to approximately 50 °C before adding 10 µl of 10 mg/ml ethidium bromide solution. The agarose solution was immediately poured into a sealed tray (Bio-RAD) with a comb placed to form wells and allowed to set at room temperature. The comb was then removed. 3 to 5 µl DNA solution of interest were mixed with 3 µl Bromphenol Blue

loading dye (containing 0.25 % (w/v) xylene cyanol, 0.25 % (w/v) bromophenol blue and 50 % (v/v) glycerol) before loading into gel wells and run with 0.5x TBE buffer at 100 Volts direct current in a SUB DNA cell (Bio-RAD). Bromophenol blue (3',3",5',5"-tetrabromophenolsulfonphthalein) carries a slight negative charge at moderate pH, therefore migrating in the same direction as DNA in the gel (at 1 % agarose at the same rate as approximately 500 bp DNA fragments). Xylene cyanol is similarly used to indicate the progress of DNA separation during gel electrophoresis (at 1 % agarose migrating at the same rate as approximately 4,000 bp DNA fragments).

DNA separation was visualised and documented using a Gel Doc 1000 single wavelength (312 nm UV-B) mini-transluminator (Bio-RAD Laboratories Ltd.) and a Charged Coupled Device (CCD) camera using a Wratten 2a filter and 2x magnification lens.

2-8.6 Purification of DNA from gel bands

DNA was purified from agarose gel using the GFX purification kit (GE Healthcare). Therefore a narrow gel slice containing ethidium bromide stained DNA of the size of interest was cut with a sterile scalpel under UV light. The gel slice was transferred to a pre-weighted 1.5 ml microcentrifuge tube, which was then weighted again to determine the net weight of the gel slice. The slice was subsequently cut into several smaller pieces and 10 µl capture buffer per 10 mg of gel was added to the tube. After vigorous vortexing the sample was incubated at 60 °C for 15 minutes during which the agarose melted completely. The resulting solution was spun briefly for collection at the bottom of the tube and then transferred to a GFX column seated in a new collection tube. Following incubation at room temperature for 1 minute to allow DNA to bind to the glass fibre matrix the sample was spun at 13,000 rpm for 30 seconds and the flow-through discarded. 500 µl wash buffer were then applied to the column, which was subsequently spun again and the flow-through discarded. The column was then placed into a new 1.5 ml microcentrifuge tube and the purified DNA was eluted by addition of 10 to 50 µl of double distilled water (depending on the size of DNA band) directly on top of the matrix. Incubation at room temperature for 1 minute was followed by centrifugation at 13,000 rpm for 1 minute.

2-8.7 Blue/white colony selection

PCR2.1 vector (Invitrogen) enabled screening of successful cloning reactions (section 2-11.1) through the colour of the bacterial colonies. The molecular mechanism for blue/white screening is based on the Lac operon and briefly explained here. PCR2.1 contains the ß-galactosidase gene (Lac Z) within the multiple cloning site (MCS). Cloning in pCR2.1 was performed with isopropyl ß-D-1-thiogalactopyranoside (IPTG) present in the medium, which functions as the inducer of the Lac operon in the absence of lactose. If no cloned insert was present in the pCR2.1 vector (which would interrupt the Lac Z gene) IPTG triggered the expression of ß-galactosidase by induction from the T7 promoter. The enzyme then processed 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) within the medium (a colourless modified galactose sugar that is metabolized by β-galactosidase). The hydrolysis of colourless X-gal by the β-galactosidase caused the characteristic blue colour indicating that the colonies contained unmodified vector. White colonies indicated insertion of HLA sequence and thus loss of the cells' ability to hydrolyse the marker. An example of colonies grown under conditions for blue/white screening is shown in Figure 2-8.

Figure 2-8 Blue/white colony screening

This picture shows part of a Petri dish with agar, on which bacteria were grown under conditions suitable for blue/white screening. Several colonies of blue or white appearance can be observed.

Growth of untransformed cells was suppressed by the presence of Kanamycin in the growth medium. A Kanamycin resistance gene on pCR2.1 allowed successfully transformed bacteria to survive despite the presence of the antibiotic.

2-8.8 Glycerol stock

Glycerol stocks were prepared from bacterial hosts carrying engineered DNA vectors. Therefore single colonies were picked from plates, on which they had been grown on selective medium overnight and were subsequently grown with the appropriate selective liquid medium overnight. 0.5 ml of the overnight culture was then mixed with the same volume of glycerol in a screw cap microcentrifuge tube for storage at -80 °C.

2-8.9 Polymerase chain reaction (PCR)

Conventional PCR was performed on a PTC-200 Peltier Thermal Cycler (MJ Research INC.) using the conditions described in the relevant sections of this chapter. Therefore agarose gels or capillary electrophoresis were used for detection of PCR product sizes at the final phase of the PCR reaction.

Quantitative PCR was used for measuring the kinetics of PCR amplification during the early exponential phase of the reaction when (assuming 100 % reaction efficiency) doubling PCR products accumulate at every reaction cycle. It was performed in 96 well optical reaction plates (ABI Prism) using the spectrofluorometric thermal cycler ABI 7500 Real Time PCR instrument (Applied Biosystems) as described below for calculation of the quantitatively related amount of starting material and sensitive detection of variability between samples.

Therefore either an intercalating dye (SYBR Green I) or fluorescent Taqman probes were used. SYBR Green dye bound to the minor groove of double stranded DNA (increasing its fluorescence over a hundred fold upon binding) and as more amplicons were produced during the PCR reaction, the fluorescent signal increased.

Whereas SYBR Green bound to any double stranded DNA molecule, the use of fluorescent probes allowed specific detection of amplification of pre-determined targets. Taqman probes were designed to anneal to a specific sequence of the template between the forward and reverse primers used for amplification of the target. They contained a high-energy dye (reporter) at the 5' end and a low-energy molecule (quencher) at the 3' end. Excitation of the intact probe resulted in the reporter dye's emission being suppressed by the quencher dye in close proximity due to fluorescence resonance energy transfer (FRET). During PCR, the 5'-exonuclease activity of the *Taq* DNA polymerase cleaved the probe that sits in the path of the enzyme. This stopped the transfer of energy from the reporter to the quencher that were no longer in proximity thereby decreasing the quencher signal and increasing the reporter signal with the latter

captured by the sequence detection instrument. Using fluorescent probes, an increase in the product targeted by the reporter probe at each PCR cycle causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter. Quantitative PCR is often performed on cDNA generated from the same quantities of RNA or with internal controls that allow for variations in the amount of RNA. For purposes for this project, however, absolute quantification of transcripts was not necessary and therefore cDNA generated from the total amount of RNA extracted with carrier was used to enable use of small cell numbers for quantitative PCR.

Analysis was performed using the ABI 7500 SDS software Version 1.2 (Applied Biosystems). The detection system plotted the level of fluorescence detected in the different wells as a function of the PCR cycle number, which allowed verification of successful PCR amplification. In addition a minimum threshold of detection was set, depending on the fluorescence detected in positive and negative control samples. An illustration is shown in Figure 2-9.

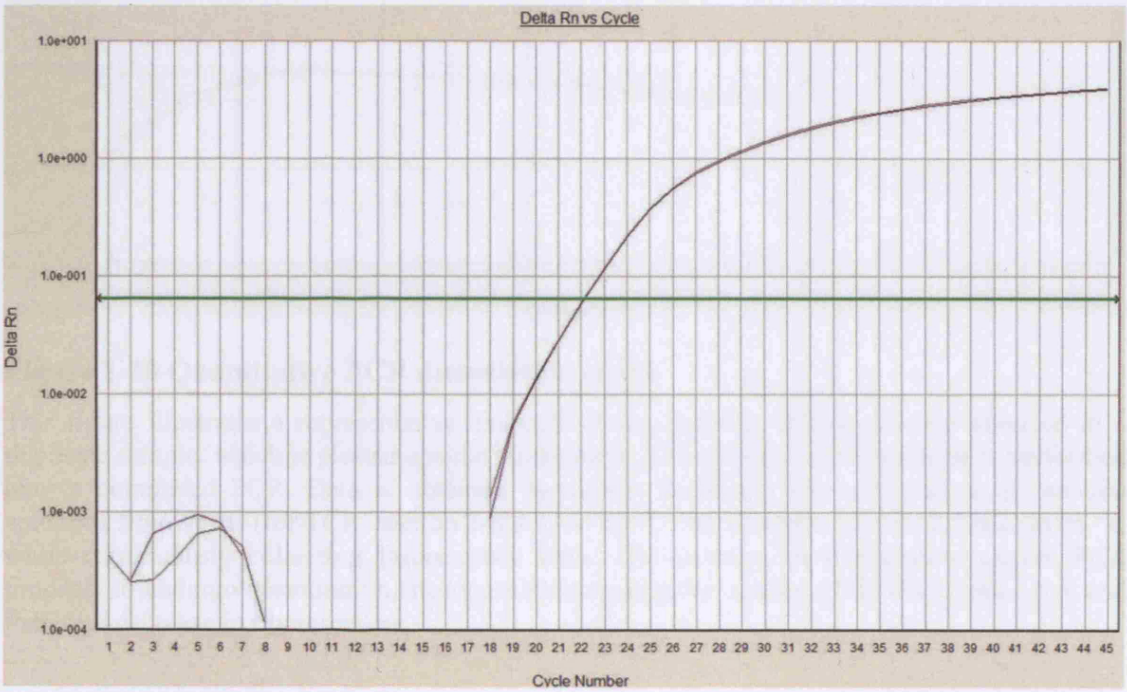


Figure 2-9 Quantitative PCR amplification plot

This figure illustrates the level of fluorescence (Delta Rn) detected in a representative duplicate sample that was plotted as a function of PCR cycle number. The threshold of detection that was set for this assay is shown at 0.0645 Delta Rn (green line).

For assays using SYBR Green I, a dissociation curve was plotted for each sample. A representative example is shown in Figure 2-10. As temperature increases, PCR amplification products eventually dissociate into single strands. Dissociation to single-stranded nucleic acids is known to occur rapidly at a certain temperature, which is called melting temperature (Tm). Using dissociation curves, specific PCR amplification can be detected as a curve with a single peak whereas nonspecific amplifications, including those of primer dimers, can be detected as a curve with multiple peaks resulting from different melting temperatures of the PCR products.

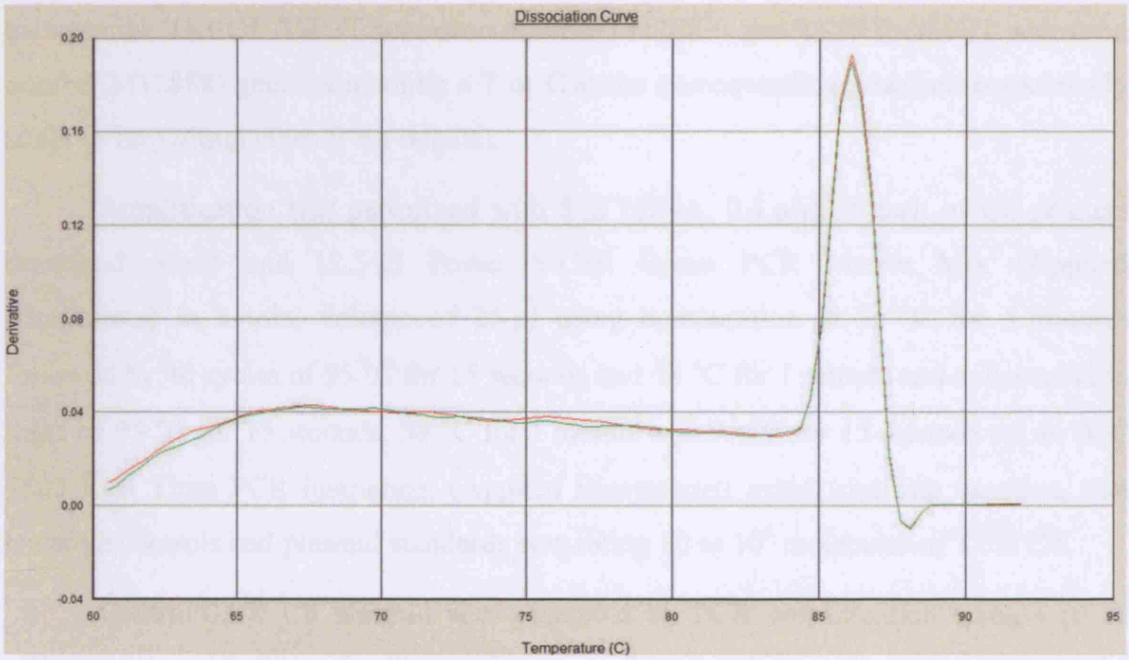


Figure 2-10 Quantitative PCR dissociation curve

This figure illustrates a representative example of the derivate of fluorescence detected in a duplicate sample, which is plotted against temperature. Dissociation curve analysis is performed after a completed PCR. Data is obtained by slowly increasing the temperature of reaction solutions from 58 °C (for TCR, section 2-8.9.1) or 60 °C (for albumin, section 2-8.9.2) to 95 °C while continuously collecting fluorescence data. The increase in temperature causes PCR products to undergo denaturation, a process accompanied by release of SYBR Green I dye and therefore decrease in fluorescence.

The detection system determined the threshold cycle value (Ct), which is the threshold cycle at which significant fluorescence is first detected above background. The Ct value was then translated into a quantitative result by the construction of a standard curve (refer to Figure 4-6 on page 273 for an example).

2-8.9.1 T cell receptor (TCR) Cß mRNA quantification

Several primers were designed and tested for amplification of TCR Cß mRNA. The primer pair chosen for final amplification of a 195 bp product was Cß forward 5' TGA CTC CAG ATA CTG CCT GA 3' (Alta Biosciences) and Cß reverse 5' CGT **CT**G ACA CCG AAR TGG AG 3' (Alta Biosciences). The latter spans the boundary between exons 1 and 2 (marked in bold) thereby avoiding amplification from genomic DNA. The "R" at position 15 indicates that the primer was used at a 50:50 ratio of primers containing either A or G at this position, which accounts for differences between the TRBC1 (IMGT accession number M12887) and TRBC2 (IMGT accession number M12888) genes containing a T or C at the corresponding positions respectively (refer to http://imgt.cines.fr for details).

Amplification was performed with 5 µl cDNA, 0.4 µM of each of the primers described above and 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 µl using denaturation at 95 °C for 5 minutes followed by 40 cycles of 95 °C for 15 seconds and 58 °C for 1 minute and a dissociation stage of 95 °C for 15 seconds, 58 °C for 1 minute and 95 °C for 15 seconds on an ABI 7500 Real Time PCR instrument (Applied Biosystems) using triplicate samples, non template controls and plasmid standards containing 10 to 10^8 molecules of TCR Cß.

Control TCR Cß plasmid was generated by PCR amplification using 1 µl of cDNA generated from healthy volunteers, 2 mM $MgCl_2$ (Invitrogen), 0.4 mM deoxynucleotide-triphosphates (dNTPs), 0.4 µM of Cß forward and reverse primers each and 0.53 units platinum *taq* (Invitrogen) with 1x platinum *taq* buffer (Invitrogen) in a final volume of 25 µl. PCR conditions were 95 °C for 1 minute followed by denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds and primer extension at 72 °C for 5 minutes for 30 cycles followed by a final polymerisation step of 10 minutes at 72 °C. Resulting PCR products were used for ligation into pCR2.1 TOPO-TA cloning kit (Invitrogen), transformation of TOP10F' cells (Invitrogen) and plasmid DNA extraction as described in section 2-11.1.

The weight of a TCR Cß DNA plasmid was calculated by multiplying the molecular weight of 2 nucleotides (660 g/*mol*) with the total amount of base pairs contained in each plasmid (3,931 bp vector lengths plus 195 bp PCR product add up to 4,126 bp of plasmid) resulting 2,723,160 g/*mol*. As 1 *mol* has $6x10^{23}$ molecules, the weight of DNA plasmid was divided by $6x10^{23}$ to establish that 1 molecule of DNA

plasmid weights $4.5386x10^{-18}$ g. The plasmid DNA was linearised by digestion with 8 µl PstI (Roche) and 8 µl SuRE/Cut buffer H (Roche) at 37 °C overnight and used for absorbance measurement at 260 nm to establish the concentration of original plasmid DNA solution as 310 ng/µl, therefore $310x10^{-9}$ g/µl. This number was divided by the established weight of one molecule of plasmid DNA to determine a weight of $6.8303x10^{10}$ molecules/µl as the molecular concentration of original plasmid DNA solution, which was subsequently diluted to obtain standards containing 10 to 10^8 molecules of TCR Cß.

Control amplification of 105 bp amplicons was performed from the transferrin receptor gene (TfR, CD71, P90), which is constitutively low expressed in resting T cells. Therefore 5 µl cDNA sample was amplified with 0.5 µl ROX (5-carboxy-X-rhodamine) dye, 0.5 units platinum *taq* (Invitrogen), 3.5 mM $MgCl_2$, 0.2 mM dNTPs and 2.5 µl of pre-designed and validated primer/probe mix (with VIC dye label, minor groove binder (MGB) and non-fluorescent quencher (NFQ) located in exon 14) (Applied Biosystems) in a total volume of 25 µl with 1x platinum *taq* buffer (Invitrogen) in triplicates along with non-template controls and plasmid standards containing 10 to 10^8 molecules of transferrin receptor.

Standards were prepared as described for TCR Cß using an initial PCR amplification with 2.5 µl of CD71 primer/probe mix (Applied Biosystems) instead of Cß primers and an annealing temperature of 65 °C.

Thermal cycling was performed using denaturation at 95 °C for 5 minutes followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 1 minute on an ABI 7500 Real Time PCR instrument (Applied Biosystems).

The TfR gene was chosen as the preferred template for control amplifications after comparison with a variety of different other constitutive genes. These other genes were considered not appropriate for quantitative PCR for various reasons. PCR amplification of a 285 bp template of the ß Actin gene using the primers 5' TCA TGA AGT GTG ACG TTG ACA TCC GT 3' and 5' CTT AGA AGC ATT TGC GGT GCA CGA TG 3' resulted in consistent problems with non specific amplifications detected in non-template controls. PCR amplification from other genes resulted in dissociation curves with multiple peaks suggestive of unspecific amplification. Examples include the amplification from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using the primers 5' GTC AGC CGC ATG TTC TTT T 3' and 5' ATC GCC CCA CTT

GAT TTT 3', which resulted in a dissociation curve with two different peaks originating from amplification of a 319 bp product in addition to amplification of a 265 bp product from two different mRNA splice patterns. Multiple dissociation curves were also obtained during PCR amplification from a 345 bp template of hypoxanthine-guanine phosphoribosyltransferase (HPRT) using the primers 5' CAG TCA ACA GGG GAC ATA AAA 3' and 5' TGA ACT CTC ATC TTA GGC TTT G 3'.

2-8.9.2 T cell receptor excision circle (TREC) quantification

TREC analysis was performed on 4 samples from patient 28 that were obtained at day 64, day 113, day 120 and day 127 post transplantation. Therefore triplicate samples of $5x10^5$ PBMC were spun at 13,000 rpm for 1 minute and the supernatant removed. 50 µl of a 100 µg/ml proteinase K solution (Sigma) were added to the cell pellet and vortexed before incubation at 56 °C for 2 hours. This resulted in digestion of proteins and inactivation of nucleases for preservation of highly native, undamaged DNA. The solution was then vortexed again and incubated at 95 °C for 15 minutes to inactivate proteinase K. The sample was subsequently spun at 13,000 rpm for 1 minute to collect the resulting DNA lysate at the bottom of the tube and stored at -70 °C until use for TREC analysis.

For TREC analysis, 5 µl cell lysate was amplified with 0.5 units Platinum *Taq* (Invitrogen), 3.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTPs (Bioline), 0.4 µM TREC forward and TREC reverse primers (Sigma Genosys) each, 0.4 µM TREC probe (Sigma Genosys) and 0.5 µl of the 25 µM ROX reference dye (USB Corporation) in Platinum *Taq* buffer (Invitrogen) in a final volume of 25 µl.

Primers did amplify signal joint TREC (section 1-8.3) and were of sequences published by Douek and colleagues (Douek *et al.*, 2000). The TREC forward primer was 5' CAC ATC CCT TTC AAC CAT GCT 3', the TREC reverse primer was 5' GCC AGC TGC AGG GTT TAG G 3' and the TREC probe was 5' FAM (6-carboxyfluorescein) ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT TAMRA (N,N,N',N'-tetramethyl-5-carboxyrhodamine) 3'.

Thermal cycling was initiated with 2 minutes of incubation at 50 °C, followed by a first denaturation step at 95 °C for 10 minutes and then 40 cycles of 95 °C for 30 seconds and 60 °C for 40 seconds on an ABI 7500 Real Time PCR instrument (Applied Biosystems) along with triplicate plasmid standards (provided by A. Knight,

ANRI) containing 10 to 10^8 molecules of TREC (standard curve slope -3.85, intercept 46.10, coefficient of determination $R^2 = 0.998769$).

PBMC from the samples mentioned above were simultaneously used for surface staining with CD3 and CD4 (the patient had not recovered $CD8^+$ T cells) as described in section 2-5 for calculation of TREC numbers per $CD4^+$ T cells.

Lysates were also tested by quantitative PCR for albumin, which confirmed the presence of similar quantities of the constitutive gene albumin in all cell lysates tested. This was performed by amplification of 5 µl lysate with 0.5 µM primer 1 (GCT GTC ATC TCT TGT GGG CTG T) and primer 2 (ACT CAT GGG AGC TGC TGG TTC) each and 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 µl using denaturation at 95 °C for 5 minutes followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 1 minute and a dissociation stage of 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds on an ABI 7500 Real Time PCR instrument (Applied Biosystems).

2-9 Synthetic peptides

All peptides were purchased from Alta Biosciences, UK, and were at least 80 % pure. They were dissolved in dimethylsulfoxide (DMSO), their concentration determined by bicinchoninic acid (BCA) Protein Assay (PIERCE) (section 2-10) and stored at -20 °C. Proteins were diluted to the required quantity in ≤50 % DMSO and stored at 4 °C for up to 2 weeks before use for tetramer generation or stimulation experiments.

CMV derived peptides used during the main experiments described throughout this thesis are listed in Table 2-5.

Peptide position	Length	HLA restriction	Sequence
pp65 (123-131)	9	HLA-B*3501	IPSINVHHY
pp50 (245-253)	9	HLA-A*0101	VTEHDTLLY
pp65 (363-373)	11	HLA-A*0101	YSEHPTFTSQY
pp65 (341-349)	9	HLA-A*0101 [1] HLA-A*2402	QYDPVAALF
pp65 (113-121)	9	HLA-A*2402	VYALPLKML
pp65 (495-503)	9	HLA-A*0201	NLVPMVATV

Table 2-5 Peptides used in this study

This table lists peptides and the respective HLA restriction in which they have been studied within this thesis. [1]Restriction of this peptide by HLA-A*0101 was reported previously but not confirmed in this study.

Details of the peptides' description in the literature are shown in the relevant result chapters.

2-10 Estimation of peptide and protein concentrations

The BCA Protein Assay (PIERCE) is based on the reduction of Cu^{2+} to Cu^{1+} in an alkaline medium, which is detected by bicinchoninic acid (BCA). Chelation of copper with protein in an alkaline environment results in formation of Cu^{1+}. Chelation of two molecules BCA with one Cu^{1+} result in formation of a purple coloured reaction product, which absorbs at 562 nm depending on the protein concentration. The rate of BCA colour formation is dependent on the incubation temperature and protein concentration. The latter can be estimated by means of a standard curve.

Briefly, albumin standards containing bovine serum albumin (BSA) at a concentration of 2 mg/ml in 0.9 % (v/v) saline and 0.05 % (w/v) sodium azide (PIERCE) were diluted in water to 0.5 µg, 1 µg, 2 µg, 4 µg and 8 µg in a total volume of 500 µl each. Standards along with a negative control containing water only and samples at three different dilutions in water, each in a total volume of 500 µl, were incubated with 500 µl of freshly prepared BCA working reagent (mix of 50 parts of reagent A containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH, 48 parts of reagent B containing 4 % bicinchoninic acid in water and 2 parts of reagent C containing 4 % cupric sulphate pentahydrate in water). After incubation in a heating block preheated to 60 °C for 60 minutes, samples were read in 1 ml plastic cuvettes (Jencons PLS) on a spectrophotometer (Jenway) at 562 nm against water. The optical density of water was subtracted from all samples and protein concentration was determined using a standard curve generated from optical density of albumin standards.

2-11 Synthesis of tetramers

2-11.1 Cloning of HLA-A24 heavy chain cDNA

Cloning of HLA-A24 heavy chain required optimisation of different variables during molecular experiments with the final settings used described below.

Escherichia coli (E. coli) JM109 K12 derivate host cells (Stratagene) were grown on plates with selective M9 Medium (90 mM $Na_2HPO_4x1H_2O$, 22 mM KH_2PO_4, 8.5 mM NaCl, 18 mM NH_4Cl with addition of 0.4 % (w/v) glucose, 1 mM thiamine, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$ and 1.5 % (w/v) agar) by overlaying 50 to 200 µl cell

culture on different Petri dishes (BD) containing the selective culture medium with 1.6 % (w/v) bacterial agar (Gibco BRL). Single colonies were picked and incubated in 2XYT medium (16 g tryptone (Gibco BRL), 10 g yeast extract (Oxoid) and 5 g NaCl (BDH) per litre) shaking at 37 °C until they reached an OD_{600nm} of 0.9. Then 25 µl of JM109 culture were grown with 5 µl M13 bacteriophage containing the HLA-A*24020101 sequence (provided by Dr. Ann-Margaret Little, Tissue Typing Laboratories, ANRI) in 5 ml 2XYT medium overnight. Cells were spun at 3,300 rpm and pellets used for DNA extraction using a Miniprep Kit (Stratagene).

DNA was used for polymerase chain reaction (PCR) to amplify the extracellular region of the HLA-A*2402 nucleotide sequence. Regions encoding the trans-membrane and intracellular portion of the HLA-A24 protein were excluded because these hydrophobic regions can complicate solubilisation and refolding of the expressed protein in later steps during the generation of tetramers and they are not needed for the molecule to bind to the T cell receptor. Primers were designed to amplify HLA-A*24020101 (IMGT/HLA accession number HLA0050) region 65 to 909 whereby positions 65-78 and 897 to 909 were modified leaving the regions spanning exon 2 (starting at 74) to exon 4 (ending at 895) largely intact. PCR is illustrated in Figure 2-11. The primers used were designed to add an Nco I (including the start codon) or BamH I restriction site to either site of the PCR product (primer "131" containing a Nco I restriction site (blue) and a codon encoding methionine marking the start of transcription (underlined): 5' GCA CCA TGG GCT CTC ACT CCA TG 3'; primer "140" containing a BamH I restriction site (orange): 5' CTG GGA AGA CGG ATC CCA TCT CAG GGT 3'). Primers were purchased from Amersham Pharmacia Biotech.

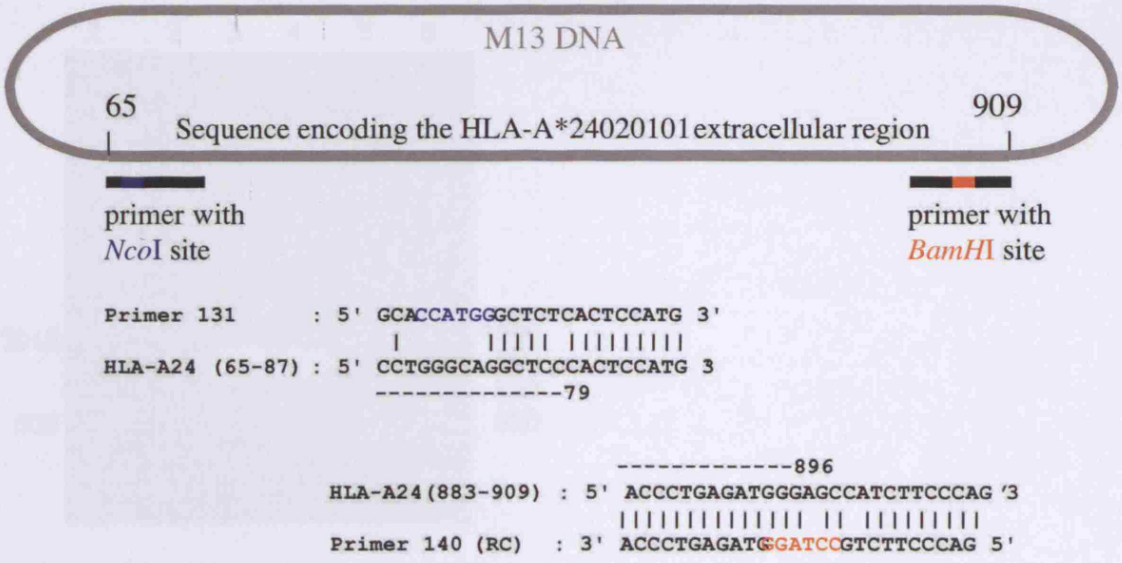


Figure 2-11 Illustration of HLA-A24 PCR amplification primers that add Nco I and BamH I restriction sites

This figure illustrates the sites of amplification of the extracellular region of HLA-A24 (from residue 65 to 909) from M13 DNA containing the HLA-A24 sequence. The sites where primers modify the original sequence with Nco I (blue) and BamH 1 (orange) restriction sites are illustrated in the upper part of the graph whereas alignment of these primers (primer 140 shown in reverse complement) with the HLA-A24 nucleotide sequence is shown in the lower part of the graph.

Amplification was performed in 50 µl reactions using 2.5 U High Fidelity Platinum *Taq* (Invitrogen) to circumvent the occurrence of mutations in the extracellular region of HLA-A24. Other reagents included 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (Bioline), 2 mM $MgSO_4$ (Invitrogen) and 0.2 µM of both primers in 1x High Fidelity buffer (Invitrogen). The PCR amplification programme involved 94 °C for 2 minutes followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 90 seconds at 68 °C and a final cycle of 30 seconds at 94 °C, 30 seconds at 55 °C and 10 minutes at 68 °C.

The melting temperatures (Tm) of each primer were calculated as 64 °C for primer "131" and 67 °C for primer "141" using the formula $Tm = 61.2 + 0.41(\%GC) - 500/L$ (whereby % GC is the percentage of deoxyguanosine and deoxycytidine bases contained in the primer and L is the length of the primer). An annealing temperature of 55°C was used to account for the mismatch in primers.

PCR products were subjected to gel electrophoresis on a 0.8 % TAE (0.04 M Tris acetate, 1 mM EDTA) gel, which is demonstrated in Figure 2-12.

Figure 2-12 PCR amplification of HLA-A24 from JM109 plasmid DNA

This figure shows gel electrophoresis (0.8 % agarose, 100 Volt) of PCR products obtained after amplification of M13 DNA (containing the HLA-A*24020101 sequence) with primers 131 and 140, which was visualised by use of ethidium bromide and exposure to ultraviolet light. Lane 1: 1 kilo base (kb) DNA Ladder (Invitrogen), lane 2: PCR product from water template; lane 3 and 4: PCR product from DNA isolated from two different JM109 host cell clones; lane 5: blank; lane 6: 1 kb Plus DNA Ladder (Invitrogen). The molecular weight of the marker's relevant standards (in bp) is shown beside the gel picture.

Bands corresponding to a size of 845 bp were recovered from the gel using GFX gel band purification kit (GE Healthcare) and ligated into pCR2.1 vector using the pCR2.1 TOPO-TA cloning kit (Invitrogen) as illustrate in Figure 2-13.

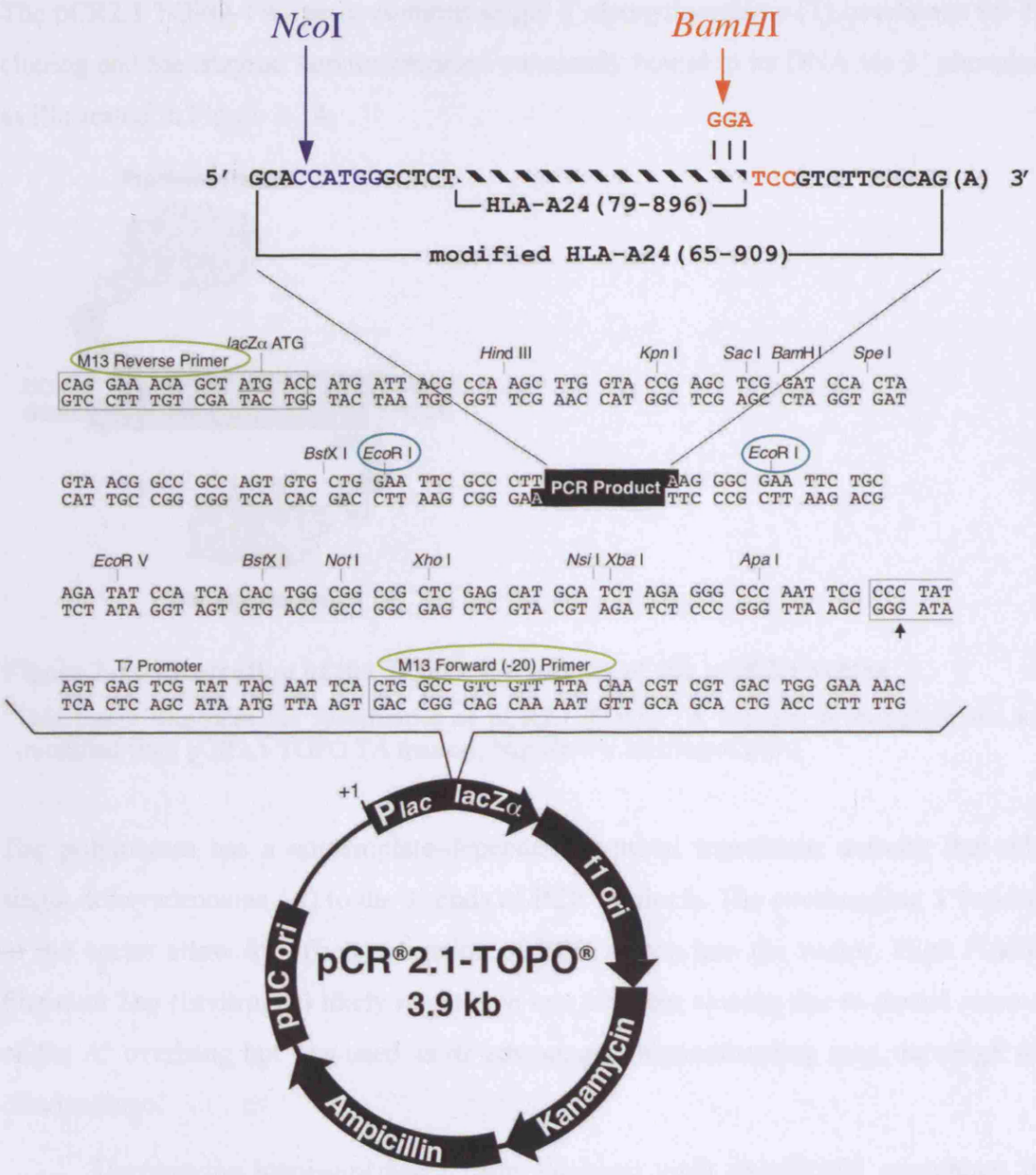


Figure 2-13 Illustration of ligation of Nco I/BamH I modified HLA-A24 insert into pCR2.1 vector

This figure illustrates the site of ligation of the Nco I/BamH I modified HLA-A24 PCR product into pCR2.1 vector (modified from a pCR2.1 TOPO map provided at http://www.invitrogen.com). EcoRI restriction sites and sites for sequencing with M13 primers that were used in further experiments are highlighted in circles.

The pCR2.1 TOPO-TA vector contains single 3'-deoxythymidine (T) overhangs for TA cloning and the enzyme Topoisomerase I covalently bound to its DNA via 3' phosphate as illustrated in Figure 2-14.

Figure 2-14 Illustration of the TOPO TA version of the pCR2.1 vector

This figure illustrates the specificities of pCR2.1 TOPO TA that are described in the text (modified from pCR2.1 TOPO TA manual, http://www.invitrogen.com)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds single deoxyadenosine (A) to the 3' ends of PCR products. The overhanging T residues of the vector allow for efficient ligation of PCR inserts into the vector. High Fidelity Platinum *Taq* (Invitrogen) likely resulted in less efficient cloning due to partial removal of the A' overhang but was used as its advantages of proofreading may outweigh this disadvantage.

The enzyme topoisomerase I from *Vaccinia* virus specifically recognises the pentameric sequence (C/T)CCTT and cleaves the phosphodiester backbone of duplex DNA after that sequence in one strand (Shuman, 1991) enabling the DNA to unwind. The energy resulting from this cleavage is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-275) of the enzyme. This bond can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, thus reversing the reaction and releasing the enzyme (Shuman, 1994). Therefore topoisomerase I functions both as a restriction enzyme and ligase. It can cleave and rejoin DNA during replication and enables faster ligation of PCR products than is possible with conventional methods. A salt solution prevents free topoisomerase from re-binding and nicking the plasmid.

Briefly, 1 µl or 4 µl of PCR product diluted in sterile water were incubated with 1 µl vector solution and 1 µl salt solution in a total volume of 5 µl at room temperature each for either 5 or 25 minutes before storage at -20 °C. No difference was observed between the four experimental variations.

Chemically competent TOP10F' cells (Invitrogen) were transformed with pCR2.1 containing HLA-A24. Therefore cells were chilled at 4 °C for 30 minutes in the presence of plasmid DNA and were then briefly heat shocked (at 42 °C for 3 minutes) causing the DNA to enter the cells. Transformed cells were grown overnight on LB plates (10 g tryptone, 5 g yeast and 10 g NaCl per L, pH 7) containing IPTG (Alexis Biochemicals), X-gal (Sigma) and 80 µg/ml Kanamycin (Sigma). Single white colonies (compare section 2-8.7) were picked and grown overnight in LB medium containing 50 µg/ml Kanamycin.

Plasmid DNA was extracted by midiprep (compare section 2-8.1) and tested for the existence of an insert by digestion with EcoRI followed by gel electrophoresis. Therefore 2 µl DNA (≤1 µg) was incubated with 1 µl EcoRI (Gibco BRL) and 1 µl SuRE/Cut Buffer H (Roche) in a total volume of 10 µl at 37 °C for 4 hours. EcoRI cuts the vector at position 284 and 302. The insert should be ligated into the vector at position 291. Therefore digest of clones with successful ligation would result in the cut of an 862 bp insert (original 845 bp product plus 17 bp flanking vector region) from the remaining vector as shown in Figure 2-15.

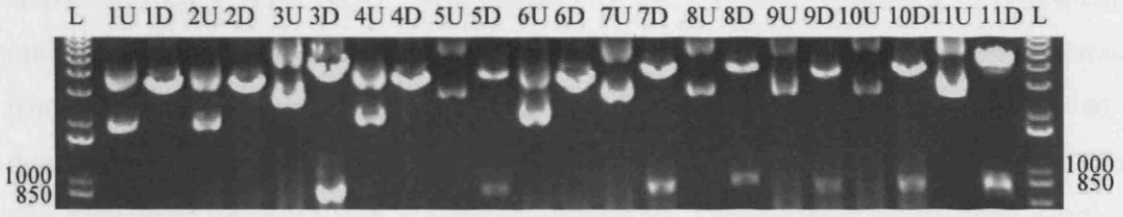


Figure 2-15 EcoRI digestion of plasmids from TOP10F' clones that had been transformed with pCR2.1-HLA-A24

This figure shows gel electrophoresis (0.8 % agarose, 100 Volt) of plasmid DNA extracted from TOP10F' clones transformed with pCR2.1-HLA-A24 and digested with EcoRI flanking the cloning site of pCR2.1 (compare Figure 2-13) Lanes L: 1 kb Plus DNA Ladder (Invitrogen); other lanes: DNA from clones 1 to 10 (11: a plasmid previously modified to contain HLA, used as positive control) that were either not digested (U) or digested with EcoRI (D). The molecular weight of relevant size standards (in bp) is shown besides the gel picture.

Bands visible from undigested plasmids from TOP10F' clones separated by gel electrophoresis (Figure 2-15) demonstrated different conformations of plasmid DNA. In

general, five conformations exist. The one running the slowest during gel electrophoresis is nicked open-circular DNA, in which one strand is cut. Linear DNA may be visible below. That confirmation has free ends, either because both strands were cut or because the DNA was linear *in vivo*. Bands running at the highest positions for cut and uncut plasmids shown in Figure 2-15 appear to be of linear conformation. Below that three more conformations may theoretically be visible. Relaxed circular DNA is fully intact with both strands uncut but has been enzymatically relaxed. Supercoiled DNA is covalently closed-circular. In this conformation DNA is fully intact (both strands uncut) with a twist built in resulting in a compact form. DNA can also be present as supercoiled denatured DNA, which has unpaired regions that make it slightly less compact, which can result from excessive alkalinity during plasmid preparation. Lower bands in Figure 2-15 lanes 1U-11U likely represent plasmids in supercoiled conformation. In lanes 1D-11D digestion with EcoRI resulted in all plasmids being linearised. An additional small band is visible in plasmids that contained the HLA-A24 insert (clones 3, 5, and 7-11).

DNA from clones that demonstrated presence of HLA-A24 inserts was also used for confirmatory sequencing (Advanced Biotechnology Centre, Imperial College, London, UK) using M13 forward and M13 reverse primers (Figure 2-13) provided with the TOPO-TA cloning kit (Invitrogen). The sequence of the M13 forward primer was 5′-GTA AAA CGA CGG CCA G-3′ and that of the M13 reverse primer was 5′-CAG GAA ACA GCT ATG AC-3′. The molarity of these primers (required to provide the optimum primer concentration for sequencing) was calculated using the formula [(number of A nucleotides x 312.2) + (number of G nucleotide x 328.2) + (number of C nucleotide x 288.2) + (number of T nucleotide x 303.2)] - 61. Nucleotide sequencing was performed using BigDye V3.1 chemistry on an Applied Biosystems 3100 capillary sequencer using 96 °C for 10 seconds, 46 °C for 5 seconds and 60 °C for 4 minutes for 35 cycles. This showed that clone 3 (compare Figure 2-15) contained the HLA-A24 sequence without mutations.

The pCR2.1 TOPO TA vector was chosen for initial cloning of HLA-A24. This is because TOPO TA features (efficient *Topoisomerase* I dependent TA cloning, presence of a cloning cassette comprising a large range of restriction sites and the presence of a gene encoding ß-Galactosidase) offered excellent conditions for efficient ligation and for different sub-cloning options if needed for later projects. The plasmid

DNA from clone 3 containing an insert of the right size and sequence was kept as stock in this vector.

DNA was subsequently digested with Nco I and BamH I for transfer into a different vector that is better suited for expression of the HLA-A24 protein for tetramer generation. Therefore 2 µl DNA (≤1 µg) was incubated with 1 µl Nco I (Gibco BRL) and 1 µl BamH 1 (Gibco BRL) and 1 µl Buffer 3 (Gibco BRL) in a total volume of 10 µl at 37 °C overnight. The HLA-A24 insert was recovered from an 826 bp band using GFX purification (GE Healthcare) for subcloning into the protein expression vector pET3d-bsp2 described below.

The vector pET8 was a gift from Dr. Studier (now commercially available from Novagen, Cambridge Bioscience, UK under the name pET3d) with a vector map shown in Figure 6-1 in the appendix. It was modified by Christina Zamoyska at the ANRI to contain a sequence coding for a biotinylation target for the enzyme biotin protein ligase (BirA) that was originally described by Schatz and colleagues (Schatz, 1993). This sequence was constructed from four complementary oligo nucleotide linkers (colours shown in accordance with Figure 2-16):

- CATGGATCGGATCCCGGCTCCGGCTCCCTGCATCATATTTTTGAAG
- CTAGCCTAGGGCCGAGGCCGAGGGACGTAGT
- CACAGAAAATTGAATGGCGCCATCGTTAAA
- ATAAAAACTTCGTGTCTTTTAACTTACCGCGGTAGCAATTTCTAG

These were made to anneal and were then ligated into an Nco I/BamH I digested pET3d vector. This resulted in complementary binding of the fragment's Bgl II overhang and the vector's BamH I digested site, thereby cancelling the BamH I restriction site at this position of the vector. The presence of the sequence for BirA substrate peptide (bsp2) in the vector resulted in a new BamH I restriction site near the Nco I restriction site that was present in the vector originally. This enabled subcloning of the modified HLA-A24 sequence into Nco I/BamH I digested pET3d- bsp2 vector, which is illustrated in Figure 2-16.

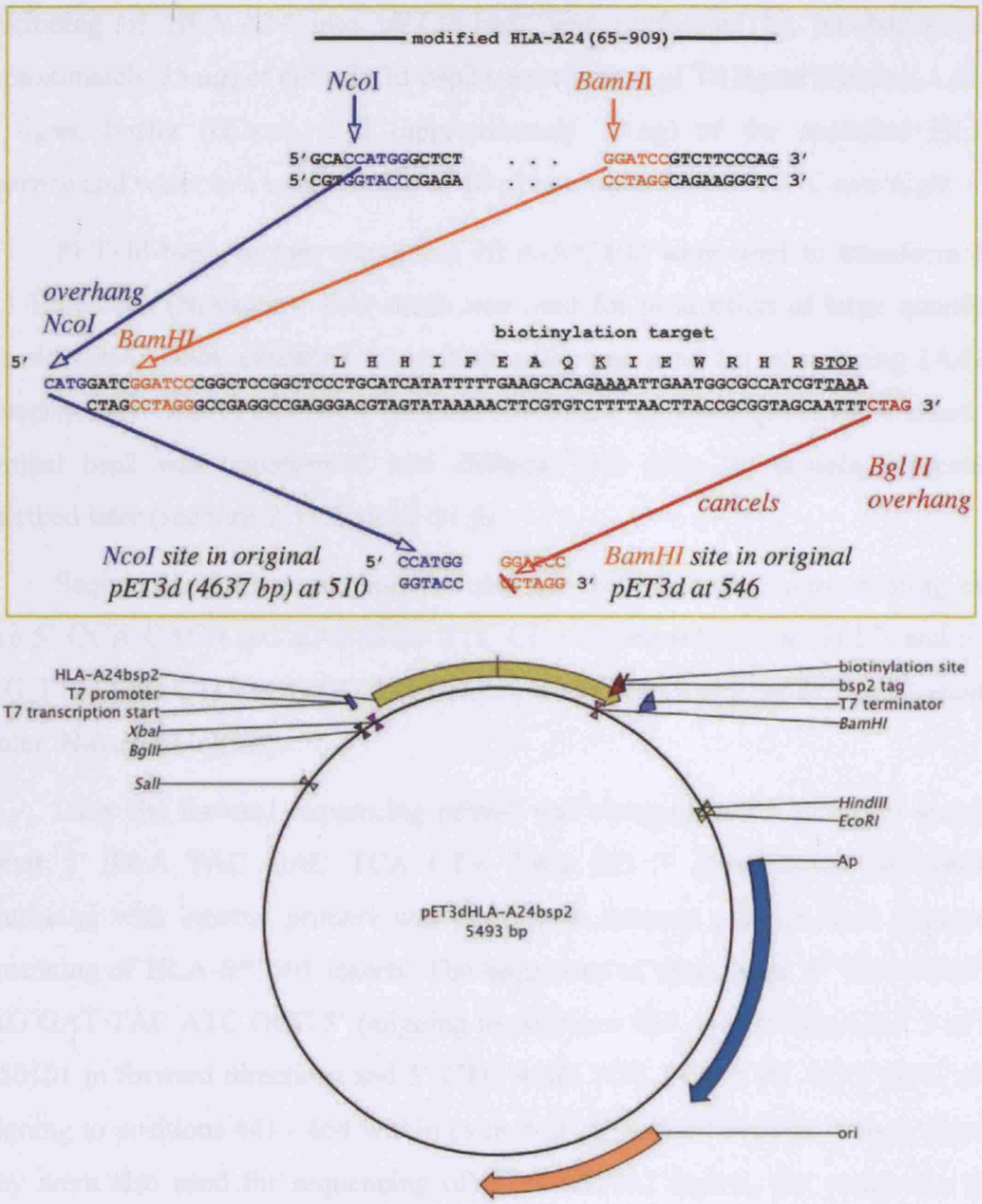


Figure 2-16 Illustration of HLA-A24 ligation into pET3d-bsp2

This figure illustrates the cloning of the 80 bp sequence encoding the biotinylation target into the Nco I/BamH I digested pET3d vector (at its original positions 546-510) via Bgl II (red) and Nco I (blue) overhangs. The amino acid sequence of the biotinylation substrate with the lysine residue (K) biotinylation target underlined is shown above the encoding bsp2 nucleotide sequence. Ligation of the HLA-A24 sequence modified to be flanked by Nco I/BamH I using the original Nco I (blue) and the newly formed BamH I (orange) restriction sites of the modified pET3d-bsp2 vector is illustrated on the top of the figure. This resulted in insertion of modified HLA-A24 (826 bp) at the positions 1 to 9 of the original bsp2 sequence with the resulting expression vector being 5,493 bp. An ATG start codon is provided within the Nco I recognition site and a TAA stop codon is provided within the bsp2 sequence (resulting in a 891 bp sequence available for translation). The vector is shown in opposite direction to the map of the original pET3d (Figure 6-1 in the appendix) so that the cloned region can be viewed clockwise (see Figure 6-2 in the appendix for complete nucleotide sequence, for alignment of sequencing primers and for the sequence of the translated HLA-A24bsp2 protein with its calculated molecular weight). Abbreviations: Ap: Ampicillin resistance gene, ori: origin of replication. This figure was generated using CLC DNA Workbench software.

Subcloning of HLA-A24 into pET3d-bsp2 was performed by incubation of 2 µl (approximately 25 ng) of cut pET3d-bsp2 vector with 1 µl T4 ligase (Gibco), 1 µl of 5X T4 ligase buffer (Gibco), 2 µl (approximately 10 ng) of the modified HLA-A24 sequence and water in a total volume of 10 µl in a water bath at 14 °C overnight.

PET-3d-bsp2 vectors containing HLA-A*2402 were used to transform *E. coli* XL1-Blue cells (Novagen). This strain was used for production of large quantities of plasmid DNA. DNA extracted from these cells was used for sequencing (Advanced Biotechnology Centre) and DNA with the confirmed sequence of the HLA insert and C terminal bsp2 was transformed into different host cells for protein expression as described later (sections 2-11.3 and 2-11.4).

Sequencing primers binding the external section of the vector cloning cassette were 5' GGA GAC CAC AAC GGT TTC CC 3' (forward primer "161") and 5' TGC TAG TTA TTG CTC AGC GG 3' (reverse primer "162", similar to the T7 terminator primer, Novagen) initially.

Later the forward sequencing primer was changed to T7 promoter sequencing primer 5' TAA TAC GAC TCA CTA TAG GG 3' (Invitrogen) and additional sequencing with internal primers was performed. Internal primers were designed for sequencing of HLA-B*3501 inserts. The sequences of these were 5' TAC GAC GGC AAG GAT TAC ATC GCC 3' (aligning to positions 424 - 447 within exon 3 of HLA-B350101 in forward direction) and 5' CTC AGG TCC TC**G** TTC AGG GCG ATG 3' (aligning to positions 441 - 464 within exon 3 of HLA-B35*0101 in reverse direction). They were also used for sequencing of HLA-A*2402 inserts, for which the reverse primer was mismatched at two positions (marked in bold). Designed primers were purchased from Alta Biosciences. The alignment of sequencing primers to the nucleotide sequence of HLA-A24-pET3d-bsp2 is illustrated in Figure 6-2 in the appendix.

2-11.2 Cloning of other HLA heavy chains and ß$_2$-microglobulin

The HLA-A*0201 and HLA-B*350101 constructs had been made in-house previously by Geraldine Aubert and Armando van der Horst (ANRI). They were propagated in XL1-Blue and their sequences were confirmed before use in this study. In the case of HLA-B35 the sequencing results from one colony demonstrated a single amino acid substitution (Alanine → Valine) at position 245 of the HLA chain, which was probably introduced during the initial PCR. This specific mutation in the α3 domain (exon 4) was

reported to result in expression of heavy chain with diminished CD8 interaction (Salter *et al.*, 1990, Salter *et al.*, 1989). Tetramers made from V245 heavy chain can interact with T cells with increased specificity (Bodinier *et al.*, 2000). Therefore DNA from this clone was chosen for HLA-B35 protein expression to generate HLA-B35/CMV peptide tetramer.

The HLA-A*0101 construct was a gift to the ANRI laboratory from Dr. Falkenburg (Department of Immunohaematology and Blood Transfusion, Leiden University Medical Centre, Netherlands). Wild-type HLA-A*2402 was cloned as described above and mutated HLA-A*2402 (msHLA-A*2402) was a kind gift from Prof. Sato (Sapporo Medical University School of Medicine, Japan) (described in (Sato *et al.*, 2002)).

Cloning of HLA-B*4402 and HLA-B*4403 was performed by cDNA generation of RNA extracted from EBV-transformed B cell lines WT47 (HLA-B*4402 homozygous) and PF97387 (HLA-B*4403 homozygous) cell lines provided by Dr. Steve Marsh (ANRI). Both cell lines are from the International Histocompatibility Workshop Cell Panel (accession numbers IHW9063 and IHW9027). HLA-B44 amplification was performed with primers of the sequences 5'- GGG CGT CGA CGG ACT CAG AAT CTC CCC AGA CGC CGA G -3' and 5'- CCG CAA GCT TCT GGG GAG GAA ACA CAG GTC AGC ATG GGA AC -3'. It was performed in 50 µl reactions using 2.5 U High Fidelity Platinum *Taq* (Invitrogen), 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (Bioline), 2 mM $MgSO_4$ (Invitrogen) and 0.4 µM of both primers in 1x High Fidelity buffer (Invitrogen). The PCR amplification programme involved 94 °C for 2 minutes followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 90 seconds at 68 °C and a final cycle of 30 seconds at 94 °C, 30 seconds at 55 °C and 10 minutes at 68 °C. PCR products were subsequently ligated into pCR2.1 as described for HLA-A24. However, folding of HLA-B44 protein requires an *in vitro* system very different from what is described for other HLA proteins in subsequent sections. Experiments for the generation of HLA-B44/CMV tetramer subsequently had to be discontinued (when unforeseen problems with a vector that was planned to be used for expression meant that a new project would be needed to continue this work) and are therefore not described further.

The $ß_2$-microglobulin ($ß_2m$) was cloned into the pHN1 ampicillin resistant vector and transformed into *E.coli* strain XA90 (MacFerrin *et al.*, 1990). It was kindly

donated to the ANRI laboratories by the late Professor Don Wiley (refer to (Garboczi *et al.*, 1992) for details of the construct).

2-11.3 Expression of HLA heavy chain and ß$_2$m light chain proteins using induction with IPTG

PET-3d-bsp2 vectors containing HLA-B*3501, HLA-A*0201 or wild type HLA-A*2402 nucleotide sequences, PET-3a-bsp vectors containing HLA-A*0101 nucleotide sequences or pET-21d-bsp vectors containing mutated (ms) HLA-A*2402 nucleotide sequences were used to transform the *E. coli* strain BL21(DE3) pLysS (Novagen). The wild-type HLA-A*2402 construct was additionally used to transform BLR(DE3)pLysS and HMS174(DE3)pLysS *E. coli* cells (Novagen) as will be explained later during this section. All *E. coli* strains used for transformation experiments were chemically competent.

The above-mentioned *E. coli* strains (compare Table 2-6) were used for tightly controlled protein expression in the T7 RNA polymerase-based system. In this system the target gene is cloned under the control of the T7 promoter, which is not recognised by *E. coli* RNA polymerase. Therefore virtually no expression occurs until a source of T7 polymerase is provided, avoiding production of proteins potentially toxic to the host cell that may otherwise cause plasmid instability. The strains mentioned above are lysogens of the bacteriophage DE3. Therefore they contain a chromosomal copy of the T7 RNA polymerase gene under the control of lacUV5 promoter inducible by IPTG. In pET21d (but not pET3d or pET3a), the DE3 lysogen also encodes for a Lac repressor gene that additionally represses the transcription of T7 RNA polymerase and thereby transcription from the target gene. The bacteria with pLysS designation carry a low-level expression plasmid that encodes T7 lysozyme, which binds to T7 RNA polymerase thus inhibiting transcription by this enzyme. Therefore transcription of pET encoded proteins was very minimal during the initial period of bacterial culture. T7 lysozyme is a bifunctional protein. It also cuts specific bonds in the peptidoglycan layer of the *E.coli* cell wall (Inouye *et al.*, 1973). High levels of T7 lysozyme are tolerated when produced from a cloned gene because the protein is not passed through the inner membrane to reach the peptidoglycan layer (Studier *et al.*, 1990). Presence of this protein confers an additional advantage during purification of target protein because it aids cell lysis during membrane disruption by detergents or freeze/thaw of cells. Upon IPTG induction, overproduction of the T7 RNA polymerase effectively shuts down any low level inhibition by T7 lysozyme or Lac repressor.

Expression of ß$_2$m from the pHN1 plasmid in *E.coli* strain XA90 can be induced with IPTG (Ezaz-Nikpay *et al.*, 1994) similar to *E. coli* strains using the T7 RNA polymerase-based system. Like the pET vectors used, pHN1 conferred resistance to ampicillin by carriage of the ß-lactamase gene.

E.coli clones transformed with sequences encoding HLA heavy chain or ß$_2$m were grown in 1 litre 2XYT medium supplemented with 50 µg/ml ampicillin (Sigma) and strain specific antibiotic selection shown in Table 2-6. Therefore bacteria were scraped off the top of frozen glycerol stock (compare section 2-8.8) and inoculated into 10 ml of selective medium at 37 °C shaking at 180 rpm overnight. The overnight culture was then inoculated into 1 litre of selective medium at 37 °C shaking at 180 rpm until the mid-log of the exponential growth phase (OD_{600nm} = 04. - 0.6). At that time protein expression was induced by addition of IPTG to a final concentration of 2 mM. 1 ml pre- and post-induction cell culture samples were taken for assessment of protein expression by SDS-PAGE (section 2-11.8). After growth for 4 hours, cells were harvested by centrifugation at 4,000 g for 20 minutes at 4 °C (Beckman JB-6) and purified as described in sections 2-11.6 and 2-11.7.

***E. coli* strain**	**Antibiotic selection**
BL21(DE3)pLysS	34 µg/ml Chloramphenicol
BLR(DE3)pLysS	34 µg/ml Chloramphenicol 5 µg/ml Tetracyclin hypochloride
HMS174(DE3)pLysS	34 µg/ml Chloramphenicol 200 µg/ml Rifampicin
BLR	5 µg/ml Tetracyclin hypochloride
HMS174	200 µg/ml Rifampicin
XA90	none

Table 2-6 *E. coli* strain specific antibiotic resistance used for cell culture

This table lists *E. coli* strains used for expression of heavy chain and ß$_2$m proteins and the antibiotic selection used for culture of each strain in addition to 50 µg/ml Ampicillin (all from Sigma Aldrich).

Protein expression was uncomplicated for HLA-A*0101, HLA-A*0201, HLA-B*3501 and $ß_2$m resulting in large amount of protein in form of inclusion bodies (compare Figure 2-19, page 151). HLA-A*2402 encoded protein, however, was not produced efficiently. Target proteins may interfere with gene expression or integrity of the host cell that prevents substantial amounts of target proteins to be produced. Plasmids containing toxic genes may be destabilised in the host cells. This was shown to occur by cyclic adenosine monophosphate (cAMP) mediated derepression of lacUV5 promoter (Grossman *et al.*, 1998), which can be prevented by addition of glucose (inhibiting cAMP production through inhibition of the cAMP-producing enzyme, adenylate cyclase, as a side effect of glucose transport into the cell) to culture medium. Addition of 1 % glucose in the culture medium, however, was not found to improve HLA-A24 protein expression in hosts. Alternatively bacteria were grown in medium supplemented with carbenicillin instead of ampicillin. Ampicillin resistance can be lost in cultures as the antibiotic is degraded by the ß-lactamase enzyme secreted by bacteria and the drop of pH that usually accompanies bacterial fermentation. This may be avoided by use of the related antibiotic carbenicillin, which is less sensitive to low pH. However, this approach was not found to influence the amount of HLA-A24 protein expressed either.

BL21 is the most widely used host for target gene expression. It is deficient in *lon* and *ompT* proteases that can degrade proteins during purification. BLR and HMS174 were used as alternative hosts for induction of HLA-A24 protein. They are $recA^-$ (encoding a DNA repair protein with co-protease function) derivatives that may help stabilise target plasmids containing repetitive sequences or whose products may cause the loss of the DE3 prophage. However, these hosts produced only small amounts of the protein as illustrated in Figure 2-17.

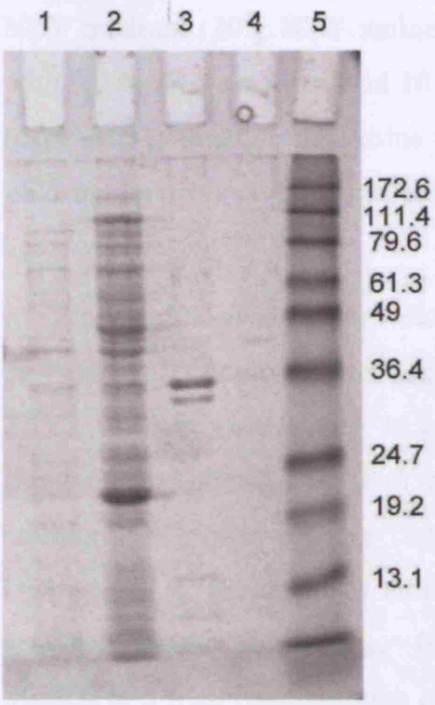


Figure 2-17 HLA-A24 expression in HMS174 (DE3) pLysS

This figure illustrates SDS-PAGE (compare section 2-11.8) separation of proteins expressed by induction of pET3d-bsp2-HLA-A24 transformed *E. coli* with IPTG. Lane 1: cell culture pre induction, lane 2: cell culture 4 hours post induction, lane 3: purified inclusion body, lane 4: empty, lane 5: BenchMark™ Pre-stained Protein Ladder (Invitrogen). The molecular weight (in kDa) of markers is shown beside the gel with the heavy chain expected below the sixth standard from the top of approximately 34 kDa.

Most HLA-A24 transformed cultures did not result in formation of inclusion bodies with a predominant protein band of the expected size (approximately 34 kDa). The best expression obtained from host strains is shown in Figure 2-17. Even in that example, however, the abundance of expression is low. Therefore alternative expression of HLA-A24 by induction with bacteriophage was tested.

2-11.4 HLA-A24 protein expression using RNA polymerase delivery by infection of hosts with bacteriophage λCE6

Introduction of T7 RNA polymerase by infection of host cells with bacteriophage λCE6 is a less convenient method than induction of DE3 lysogens by IPTG but may be a good alternative for expression of toxic genes. The method is based on the principle that λCE6 infection of hosts leads to the newly made T7 RNA polymerase to transcribe target DNA so actively that normal phage development cannot proceed.

A stock of bacteriophage λCE6 (Novagen) was prepared in ED8739 and LE392 *E.coli* hosts (Novagen) according to the manufacturer's protocol. Host cells mixed with different phage stock dilutions in top agar were grown on duplicate plates prepared with

NZY medium (10 g NZY amine, 5 g yeast extract, 5 g NaCl per litre) supplemented with 0.2 % (w/v) maltose and 10 mM $MgSO_4$. The resulting lysis plaque-forming units (pfu) were counted to determine the titer of the phage stock. Controls used were plates with top agar containing host cells without phage, plain top agar or plates without top agar.

Non-lysogenic *E. coli* clones BLR and HMS174 (Novagen) transformed with pET-3d-bsp2 containing the sequence encoding HLA-A*2402 were grown in 1 litre 2XYT medium (containing 16 g tryptone, 10 g yeast extract and 5 g NaCl per litre) supplemented with 0.2 % (w/v) maltose, 50 μg/ml ampicillin (Sigma) and strain specific antibiotic selection (Table 2-6) at 37 °C shaking at 180 rpm. After reaching $OD_{600nm} = 0.3$, glucose was added to a final concentration of 4 mg/ml and cultures grown further to an $OD_{600nm} = 0.6 - 1$. Protein expression was induced by addition of $MgSO_4$ to a final concentration of 10 mM and λCE6 stock to a final concentration of 2, 3 or $4x10^9$ pfu/ml. Pellets from pre- and post-induction samples prepared from 1 ml cell culture were stored at -20 °C for analysis by SDS-PAGE (section 2-11.8). After growth for 4 hours, cells were harvested by centrifugation (4,000 g for 20 minutes at 4 °C).

This method also resulted in small amount of HLA-A24 protein being produced. In comparison, best production of wild type HLA-A24 was achieved in IPTG induced HMS174 (DE3) pLysS hosts as illustrated in Figure 2-17. Therefore yet another alternative way of expressing this protein was exploited.

2-11.5 Protein expression from mutant HLA-A*2402 sequence

It is important to obtain large amount of soluble HLA protein for the construction of tetramers. However, conventional methods of expression and the use of variations of these to circumvent potential toxicity of HLA-A24 protein were not efficient.

Therefore low expression of the protein in *E. coli* strains may have been due to reasons other than toxicity. Sato and colleagues (Sato *et al.*, 2002) developed a mutant HLA-A24 heavy chain (msHLA-A24), in which mammalian codon usage was exchanged for silent mutated nucleotide sequences that were described to be preferentially used by *E. coli* previously (Wada *et al.*, 1992, Zhang *et al.*, 1991).

Professor Sato kindly donated the HLA-A24 expression vector for use in this project. This vector contained the HLA-A*2402 sequence region 73 - 900, which was modified to contain a start codon, restriction sites and *E. coli* preferred codons followed by a C-terminal BirA substrate (Sato *et al.*, 2002) in pET21d. After transformation into

BL21(DE3)pLysS (Novagen), expression was performed as described in section 2-11.3 and resulted in production of large amounts of HLA-A24 protein in form of inclusion bodies (compare Figure 2-18, page 149).

2-11.6 Protein purification from inclusion body preparations

Cell pellets obtained after expression of wild type heavy chains and $ß_2m$ were resuspended in 10 ml Bug Lysis Buffer (25 % (w/v) sucrose (BDH), 50 mM Tris-HCl, 1 mM EDTA, pH 8) and centrifugated at 4,000 g for 10 minutes at 4 °C. Pellets were resuspended in 10 ml B-Per Buffer (Pierce) supplemented with 25 mg lysozyme (Sigma) on ice. Membrane disruption was facilitated by freezing and subsequent thawing of cells. Samples were then sonicated (Jencons Ltd.) in 15-second bursts on ice. The inclusion bodies were pelleted at 10,000 g for 20 minutes at 4 °C. Washing steps were performed once in detergent buffer (20 mM Tris-HCl, 0.2 M NaCl, 1 % (v/v) sodium deoxycholate, 1 % (v/v) Nonidet P40, 2 mM EDTA pH 7.5) and subsequently in triton buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5 % (v/v) TritonX100, pH 8) for at least three times using centrifugation at 10,000 g for 10 minutes at 4 °C in between. Purified proteins were stored in resuspension buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulphoxide (PMSF) and 2 mM dithiotreitol (DTT) pH 8) at -20 °C for analysis using SDS-PAGE (section 2-11.9). All proteins except mutated HLA-A24 were solubilised by rolling at 4 °C overnight in freshly prepared urea buffer (8 M urea, 0.1 mM EDTA, 0.1 mM DTT, 0.01 M Tris-HCl, 0.1 M NaH_2PO_4) that had been deionised with Amberlite (Merck). Undissolved material was removed by centrifugation at 10,000 g before use for refolding (section 2-11.9).

In contrast, cell pellets from msHLA-A24 6xHis tagged protein were not washed with detergents but solubilised by resuspension in buffer A (8 M urea, 20 mM HEPES pH 8 and 50 mM NaCl) and sonication. Samples were spun at 4,000 rpm for 10 minutes at 4 °C and the supernatant collected for purification using nickel- nitrilotriacetic acid (Ni-NTA) described in section 2-11.7.

2-11.7 Mutant HLA-A24 protein purification by nickel- nitrilotriacetic acid (Ni-NTA)

The msHLA-A24 expression vector contained the HLA-A*2402 sequence and a C-terminal BirA substrate sequence followed by a thrombin recognition site in pET21d. This enabled the use of the amino acid motif hexa histidine (6xHis tag), which is encoded downstream of the HLA-A24 in the pET21d vector (Sato *et al.*, 2002). The tag can be used for specific purification of the target protein. Nitrilotriacetic acid (NTA), a chelating agent that forms coordination compounds with metal ions such as Nickel (Ni^{2+}) is used for this purpose. Proteins tagged with 6xHis tag can be separated from other proteins present in cell lysates by affinity-purification over columns containing Ni-NTA agarose beads. The affinity medium contains metal ions to which the polyhistidine-tag binds. After contaminating proteins are washed off, the purified protein can then be eluted from the column. The thrombin cleavage site (Leu-Val-Pro-Arg/Gly-Ser) at the C-terminal end of the HLA-A24-bsp construct can be used for removal of the polyhistidine tag. Following purification of HLA-A24-bsp-6xHis protein, thrombin can be used to selectively cleave and remove the purification tag from the HLA-A24-bsp protein with a high degree of specificity. This, however, did not seem necessary as the tag did not interfere with subsequent generation of functional tetramer.

Briefly, protein expressed from the msHLA-A24 construct was loaded onto 2 ml Ni-NTA-agarose resin (Qiagen, Hilden, Germany) in a glass column and equilibrated with 10 ml buffer A (8 M urea, 20 mM HEPES pH 8 and 50 mM NaCl) containing 20 mM Imidazole (BDH Laboratory supplies, UK). The sample was washed with 30 ml of the same buffer. 5 ml buffer A containing 250 mM Imidazole were then added and the purified sample collected. At this concentration of Imidazole, the 6xHis-tagged proteins dissociated because they could no longer compete for binding sites on the Ni-NTA resin resulting in elution of the HLA-A24 protein. The buffer was exchanged with 10 mM Tris-HCl pH 8 using two PD-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

The purity of the resulting protein solutions (wild type HLA heavy chain or $ß_2m$ in urea buffer or msHLA-A24 in 10 mM Tris-HCl) were tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as will be described in section 2-11.8. A representative gel is shown in Figure 2-18. The concentration of protein was assessed by BCA assay (Pierce, UK) as described in section 2-9 and aliquots were stored at -70 °C.

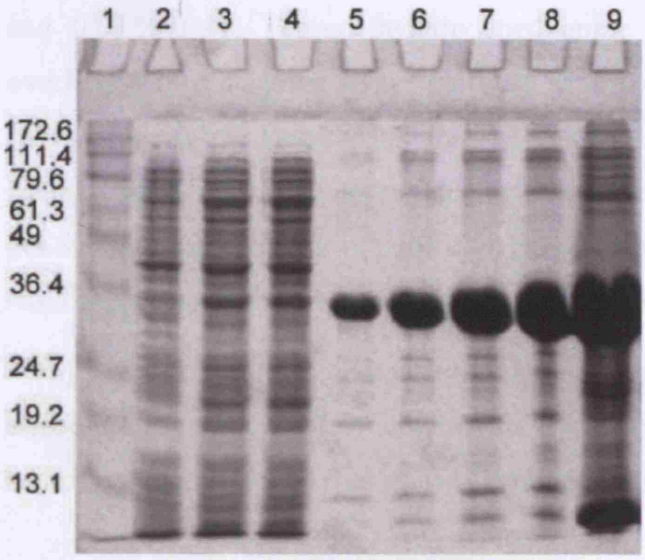


Figure 2-18 msHLA-A24 protein expression

This figure illustrates SDS-PAGE separation of proteins expressed by induction of pET21d-bsp-msHLA-A24 transformed *E. coli* with IPTG. Lane 1: BenchMark™ Pre-stained Protein Ladder (Invitrogen), lane 2: 10 µl culture sample pre induction, lane 3 and 4:10 µl culture samples at 2 and 4 hours post induction, lane 5-7: 2, 5, 10 and 15 µl inclusion body post Ni-NTA purification, lane 9: 10 µl non-purified inclusion body. The molecular weight (in kDa) of markers is shown beside the gel.

2-11.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Before separation on a gel, proteins are usually exposed to a detergent such as SDS that denatures secondary and non-disulfide linked tertiary structures and applies a negative charge to proteins, which is in proportion to their mass. Without SDS, proteins would migrate in the gel depending not only on their molecular weight but also their mass/charge ratio (compare native gel, section 2-11.13). Brief heating of protein samples in the presence of a reducing agent (such as DTT) can further denature them by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding and breaking quaternary protein structures (oligomeric subunits). Subsequently proteins migrate relative only to their size and not their shape or charge in a reducing SDS-PAGE.

The pore size of the gel used in polyacrylamide gel electrophoresis (PAGE) is determined by the concentrations of acrylamide and bis-acrylamide used. Gels consisted of a 15 % resolving lower gel (375 mM Tris-HCl pH 8.8, 50 % ProtoGel (containing 30 % (w/v) Acrylamide and 0.8 % (w/v) Bis-Acrylamide; National Diagnostics), 0.1 % (w/v) SDS (Sigma), 0.03 % (w/v) ammonium persulphate (APS) (Pharmacia Biotech)

and 0.03 % (v/v) Tetramethylethylenediamine (TEMED) (Amersham Biosciences)) overlaid with a stacking upper gel (125 mM Tris-HCl pH 6.8, 17 % ProtoGel, 0.1 % (w/v) SDS, 0.1 % (w/v) APS and 0.1 % (v/v) TEMED). These were prepared in a Mighty Small multi-gel caster (Hoefer Scientific Instruments) by pouring of the lower gel overlaid with butan-2-ol until it set (to create an even surface) followed by the upper stacking gel poured above it and insertion of a gel comp to create loading wells.

Lysis of culture supernatant samples was performed in PBS containing 1 % SDS at room temperature for 30 minutes followed by removal of undissolved material by centrifugation at 13,000 rpm prior to running on the gel.

An equal volume of protein solution or lysis samples was added to loading buffer (50 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromphenol blue, 10 % (v/v) glycerol and 100 mM DTT (Sigma)) and denatured at 98 °C for 3 minutes. Samples were then loaded onto the gel and run with electrophoresis buffer (25 mM Tris Base, 250 mM Glycin, 0.1 % (w/v) SDS, pH 8.3) at 150 volts in a Mighty Small II gel tank (Hoefer Scientific Instruments).

Gels were stained with coomassie staining solution (10 % (v/v) glacial acetic (BDH) acid and 0.25 % (w/v) coomassie brilliant blue (Sigma) in 1:1 methanol (BDH)/water) under gentle shaking for 20 minutes and excess colour was washed off with de-staining solution (16.5 % (v/v) methanol and 5 % (v/v) glacial acetic acid) that was frequently changed over a time period of at least 12 hours. Pictures were taken under white light as described in section 2-8.5. Thereafter original gels were transferred to Whatman paper that was pre-wet with detaining solution and covered with saran wrap film before drying at 80 °C for 3 hours using a horizontal vacuum gel drier (model 583, Bio-RAD) for preservation.

SDS-PAGE was used to confirm the presence of proteins produced by *E. coli*. Abundance of contaminating proteins served as a qualitative assessment of the purity of the protein preparations. A representative gel demonstrating high abundance and purity of bands corresponding to the expected HLA heavy chain and $ß_2m$ protein sizes of 34 kDa and 11.4 kDa respectively is shown in Figure 2-19.

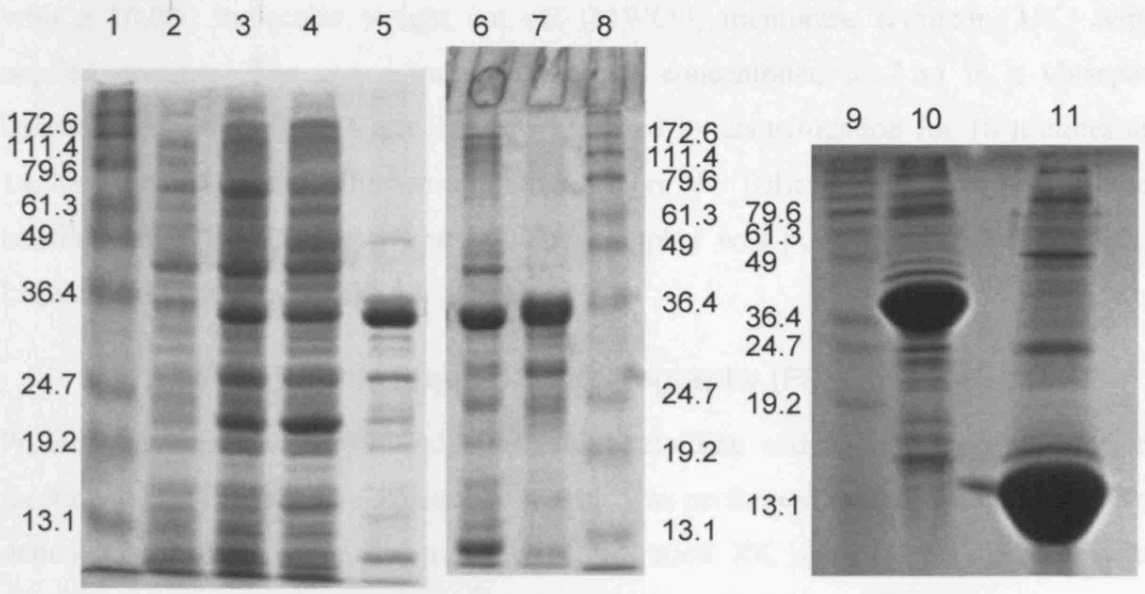


Figure 2-19 SDS-PAGE of HLA-A1, HLA-A2, HLA-B35 and ß$_2$m proteins

This figure illustrates SDS-PAGE separation of HLA heavy chain and ß$_2$m proteins expressed by induction of pET3d-bsp2 HLA transformed *E. coli* with IPTG. The left gel demonstrates expression of HLA-A*0101 protein, the middle gel demonstrates expression of HLA-A*0201 and HLA-B*3501 protein and the right gel demonstrates expression of HLA-A*0201 and ß$_2$m proteins. Lane 1, 8 and 9: BenchMark™ Pre-stained Protein Ladder (Invitrogen), lane 2: 10 µl culture sample pre induction, lane 3 and 4: 10 µl culture samples at 3 and 5 hours post induction, lane 5: 1 µl HLA-A1 inclusion body. Lane 6: 3 µl HLA-B35 inclusion body, lane 7: 2 µl HLA-A2 inclusion body, lane 10: 5 µl HLA-A2 inclusion body, lane 11: 5 µl ß$_2$m inclusion body. The molecular weight (in kDa) of markers is shown beside the gel.

2-11.9 HLA/peptide complex dilution refolding

Solution containing ß$_2$m of an amount equal to 4.8 mg was added dropwise and slowly to 200 ml pre-cooled refolding buffer (400 mM L-Arginine HCl, 100 mM Tris-HCl pH 8, 5 mM reduced glutathione, 0.5 mM oxidised glutathione, 0.5 mM PMSF, 2 mM EDTA) stirring at 4 °C. Peptide originally diluted in DMSO was diluted in water and added to an amount equalling 2 mg. Solubilised HLA heavy chain was then added in an amount equal to 6.2 mg. Heavy chain was added dropwise and stepwise over a period of at least 3.5 hours to avoid formation of aggregates.

HLA-B35 protein was observed to form aggregates easily. Therefore for refolding of HLA-B35/peptide complexes, the heavy chain was diluted to <1 mg/ml in fresh urea buffer prior to addition to 1L refolding buffer to decrease the formation of aggregates.

Refolding was allowed to proceed at 4 °C stirring slowly over 72 hours. The refolding mixture was spun for 20 minutes at 10,000 g at 4 °C to remove aggregates and filtered through a 45 µm filter if necessary before concentrating to 20 ml using a stir cell

with a 10,000 molecular weight cut off (MWCO) membrane (Amicon, UK) with applied pressure. The concentrate was further concentrated to 2 ml in a vivaspin concentrator (Sautorius). Aggregate was removed by centrifugation for 10 minutes at 14,000 rpm at 4 °C and filtration through a 0.2 µm HT Tuffryn Membrane low protein binding filter (PALL) before the refolded complex was purified using Fast Protein Liquid Chromatography (FPLC).

2-11.10 Fast Protein Liquid Chromatography (FPLC)

Protein complexes were purified after initial refolding and again after biotinylation (section 2-11.11) to remove excess biotin. This was performed by separation of proteins depending on their weight on a FPLC gel filtration XK 16/70 column packed with 120 ml superdex 75 matrix (Pharmacia Biotech) connected to an ÄKTA low-pressure automated chromatography system using Unicorn 3.10 software (Amersham-Pharmacia, UK). The purification system was set to demonstrate absorbance at 280 nm online and to collect 3 ml fractions of eluted proteins.

Initial purification was performed in Low Salt Buffer (10 mM Tris-HCl pH 8, 5 mM NaCl) as illustrated in part A of Figure 2-20 and the fractions of interest (marked refold) were concentrated to a volume of approximately 100 to 200 µl using a vivaspin concentrator (Sautorius) at 4 °C. Post biotinylation purification was performed as illustrated in part B of Figure 2-20. Therefore High Salt Buffer (10 mM Tris-HCl pH 8, 150 mM NaCl) was used. This buffer aids stabilisation of the protein complex but was not used initially because its high salt concentration can inhibit the biotinylation process. Purified post biotinylation fractions (marked refold) were concentrated to approximately 100 µl.

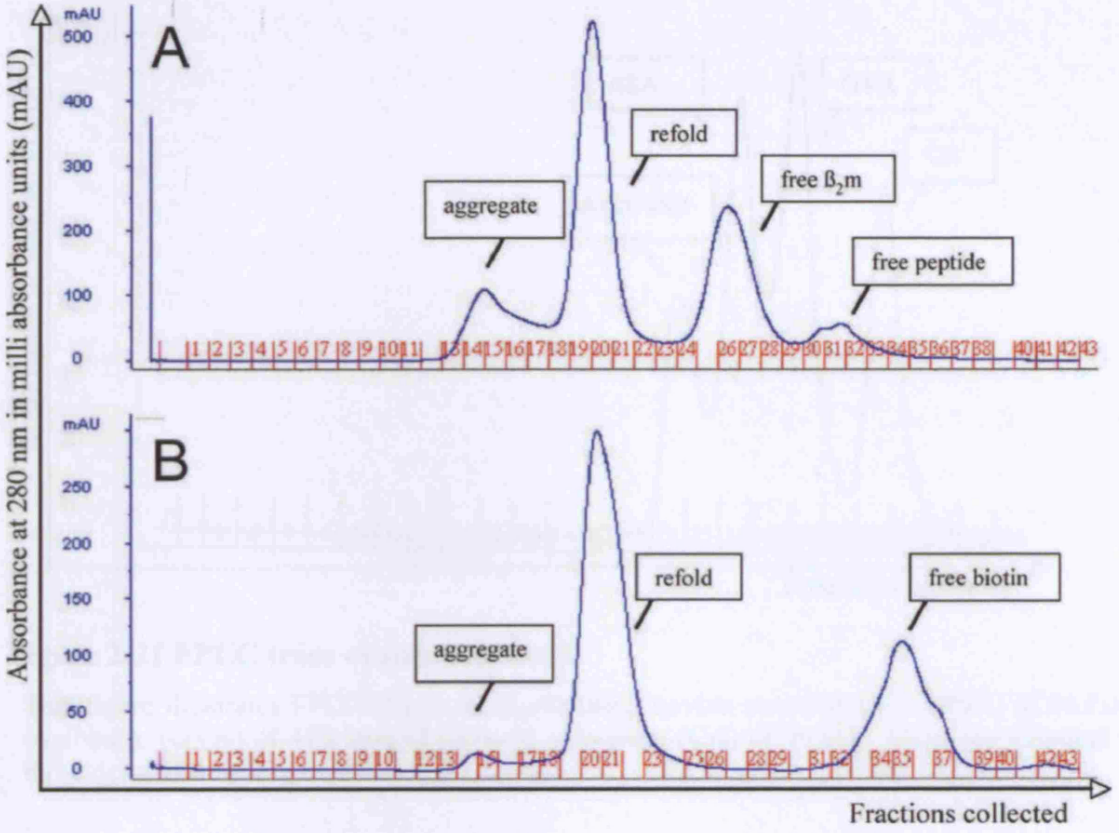


Figure 2-20 Purification of protein complexes by FPLC

This figure illustrates purification of refolded protein complex from aggregate, excess $ß_2m$ and excess peptide (and arginine originating from buffer) in part A. A further purification step from aggregate and excess biotin after biotinylation of protein complex is illustrated in part B. Absorbance at 280 nm was recorded in milli absorbance units (mAU, blue line) and collected in fractions numbered below the graph (red).

The refolded protein complex (consisting of 34 kDa HLA/bsp protein, 11.5 kDa $ß_2m$ protein and 1 kDa peptide) runs in the fractions corresponding to a size range of 35-40 kDa because charge and shape in addition to size of the complex influence its migration. The presence of refolded protein complex was confirmed by comparison with size standards, which were run after each cleaning procedure as illustrated in Figure 2-21 and additionally by testing of the refold fractions using dot blot assay (compare section 2-11.12).

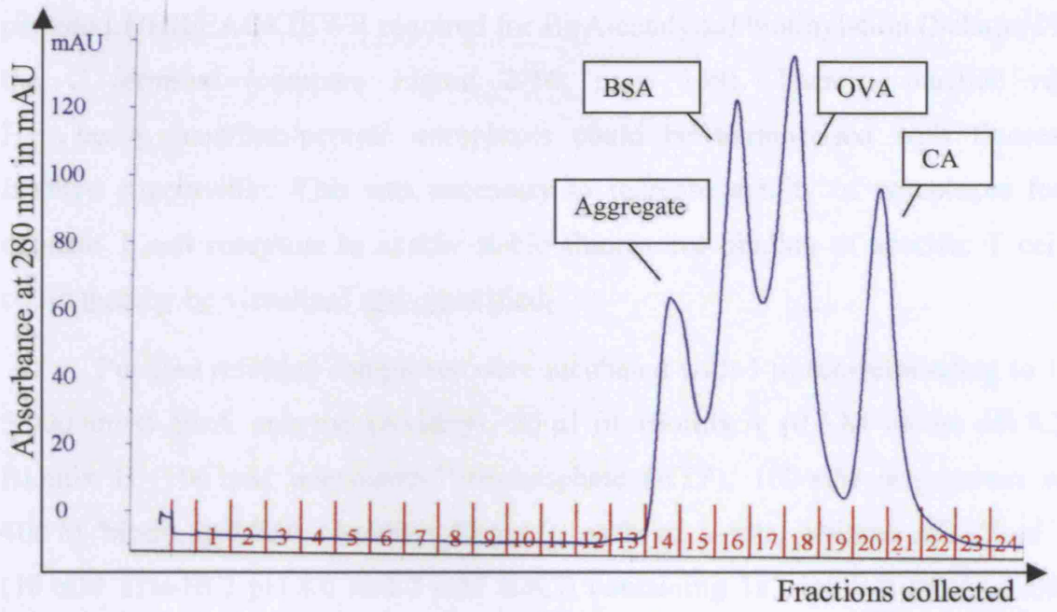


Figure 2-21 FPLC trace of size standards

This figure illustrates FPLC of size standards using bovine serum albumin (BSA) of 66 kDa, ovalbumin (OVA) of 47 kDa and carbonic anhydrase (CA) of 29 kDa. Units are identical to those described for Figure 2-20.

The column was cleaned regularly after 20 runs or before storage in 20 % ethanol. Therefore filtered and degassed solutions prepared from double distilled water were run through the column in sequential steps. 2 column volumes (240 ml) of 1 mg/ml pepsin in 0.1 M glacial acetic acid and 0.5 M natrium chloride were run in reverse flow direction at 0.5 ml/minute and incubated at room temperature over night. 2 column volumes of water were then run at 1 ml/minute followed by 60 ml of 30 % isopropanol at 0.5 ml/minute, 2 column volumes of water at 1 ml/minute reverse flow direction, 60 ml of 0.5 M NaOH at 0.5 ml/minute and 2 column volumes of water at 1 ml/minute.

If decreased performance was observed by the occurrence of less well separated peaks in FPLC traces, the column was repacked and tested for efficiency by injection of 1 ml 0.5 % (v/v) high-performance liquid chromatography (HPLC) grade acetone solution (Sigma Aldrich) in double distilled water using High Salt Buffer and calculations according to the manufacturer (typically 6,300 plates/meter).

2-11.11 Biotinylation of refolded monomers

The *E. coli* biotin holoenzyme synthetase, BirA, catalyzes transfer of biotin to the epsilon amino group of a specific lysine residue of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase. For exploitation of this process, vectors engineered to encode HLA heavy chain proteins, also incorporated a minimal substrate

peptide LHHIFEAQKIEWR required for BirA-catalyzed biotinylation (Schatz, 1993) at the C terminal (compare Figure 2-16, page 139). Thereby purified refolded HLA heavy chain/ß$_2$m/peptide complexes could be tetramerised with fluorescently labelled streptavidin. This was necessary to increase avidity of complexes for their cognate T cell receptors to enable stable fluorescent binding of specific T cells that could thereby be visualised and quantified.

Purified refolded complexes were incubated with 1 µl (corresponding to 1 µg or 5,000 units) BirA enzyme (Avidity), 25 µl of Biomix A (0.5 M bicine pH 8.3) and Biomix B (100 mM adenosine-5'-triphosphate (ATP), 100 mM magnesium acetate, 400 M biotin, pH 7.0) (Avidity, Denver), each in a total volume of 250 µl buffer (10 mM Tris-HCl pH 8.0 and 5 mM NaCl) containing 1x complete protease inhibitor (Roche) (1.5 µg/ml Chymotrypsin, 0.8 µg/ml Thermolysin, 1 mg/ml Papain, 1.5 µg/ml Pronase, 1.5 µg/ml Pancreatic Extract and 0.002 µg/ml Trypsin) at 25 °C overnight.

Thereafter monomers were immediately purified from excess biotin by FPLC as described above and protein concentration was assessed by BCA assay (section 2-10).

2-11.12 Confirmation of refolding and biotinylation using protein dot-blot and western blot

Correct folding of protein complex was confirmed by testing the refold fractions with mouse anti-conformational HLA monoclonal antibody (mAb) W6/32. This antibody was produced from hybridomas as described in section 2-4.4. It recognises a determinant present on the class I heavy chain but only when the heavy chain is in complex with ß$_2$m (Barnstable *et al.*, 1978, Ways and Parham, 1983). Additionally biotinylation of protein complex was confirmed by testing with peroxidase conjugated ExtrAvidin antibody (Sigma).

For confirmation of correct folding, Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Life Science) that had been incubated in transfer buffer (48 mM Tris base, 39 mM glycin, 0.037 % (w/v) SDS and 20 % (v/v) methanol) at room temperature for 15 minutes was used to deposit 10 µg (or a dilution thereof) of test samples onto it with a vacuum dot blotter (Hybridot Manifold, Bethesda Research Laboratories). Each dot was washed twice with 200 µl PBS. The membrane was subsequently transferred to a 50 ml Falcon tube (BD) filled with blocking buffer (PBS, 10 % (w/v) powdered milk (Marvel)) for incubation on a rolling cylinder at 4 °C overnight. After three washes for 15 minutes in PBS/0.05 % (v/v) Tween 20 (BDH

Biochemicals), the membrane was cut and samples were incubated either with 5 µg/ml W6/32 primary antibody in blocking buffer or blocking buffer only as a negative control for one hour. Membranes were then washed for a further three times with PBS/Tween 20 before incubation with 1:2,500 dilution of secondary peroxidase conjugated goat anti-mouse antibody (Sigma) for one hour. After three washes peroxidase was detected by incubation of membrane with ECL reagent (ECL reagent kit, Amersham Pharmacia Biotech) for 1 minute and immediate detection by exposure of wrapped membrane to an x-ray photographic film (MXB film, GRI autoradiography blue sensitive, Kodak) in a dark room using exposure times ranging from 45 seconds, 2 minutes, 5 minutes, 15 minutes, 1 hour to overnight to ensure detection at maximal signal to noise ratio. A representative dot blot is shown in Figure 2-22 part A.

Biotinylation was confirmed in a similar way using a single incubation with a 1:1,000 final dilution of peroxidase conjugated ExtrAvidin antibody (Sigma) instead of primary W6/32 and secondary peroxidase conjugated goat anti-mouse antibody. A representative dot blot is shown in Figure 2-22 part B.

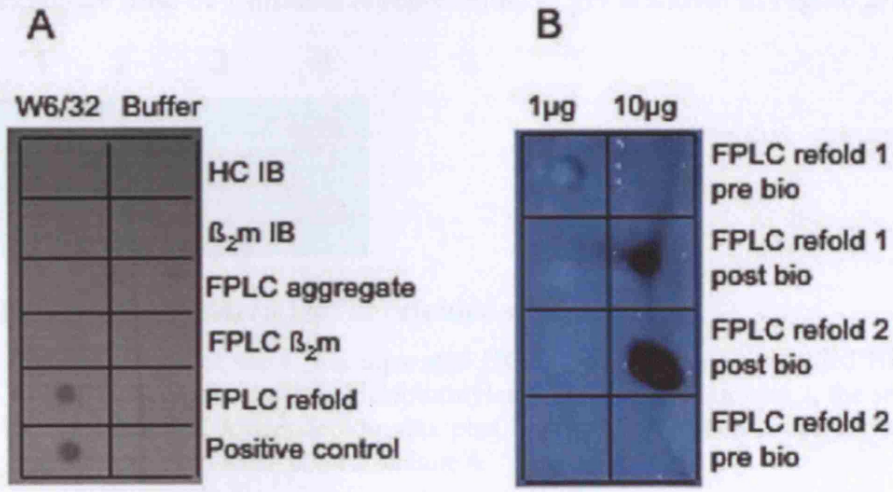


Figure 2-22 Protein dot blot of refolded complex

Part A of this dot blot illustrates probing of samples with W6/32 on the left and buffer alone on the right that were subsequently detected with (horseradish) peroxidase conjugated goat anti-mouse antibody (Promega). Samples tested were HLA heavy chain inclusion body (HC IB), ß$_2$m inclusion body (ß$_2$m IB), pre biotinylation FPLC fractions eluting at a trace position (compare Figure 2-20) correlating with aggregate (FPLC aggregate) or ß$_2$m (FPLC ß$_2$m), post biotinylation FPLC fractions eluting at a trace position correlating with refold (FPLC refold) and refolded complex that had been successfully used for generation of tetramer previously (positive control). Part B illustrates two different refolded complexes (FPLC refold 1 and 2) before (pre bio) and after biotinylation (post bio) that were detected with peroxidase conjugated ExtrAvidin antibody (Sigma).

For Western blot, a SDS-PAGE gel was prepared and 2 µg of pre biotinylation samples, post biotinylation samples and unbiotinylated or biotinylated myelin basic protein (MBP) controls (Avidity) were run along with a protein ladder as described in section 2-11.8. A membrane was incubated shortly using blocking buffer containing 0.13 M NaCl, 2.68 mM KCl, 24.77 mM Tris Base, 0.05 % (v/v) Tween 20 and 5 % (w/v) powdered milk, and the SDS-PAGE gel was immediately blotted onto that membrane using transfer buffer containing 25 mM Tris Base, 200 mM glycin and 20 % (v/v) methanol at 200 mA at 4 °C for 2 hours using a vertical Western blot apparatus (model Criterion, Bio-RAD) that was assembled in a cold room.

The membrane was then washed with Tris buffered saline (TBS)/0.05 % (v/v) Tween 20 and subsequently incubated in blocking buffer containing a final 1:8,000 dilution of peroxidase conjugated ExtrAvidin antibody (Sigma) for one hour. After extensive washing in TBS/Tween 20, peroxidase was detected by incubation of membrane with ECL reagent (ECL reagent kit, Amersham Pharmacia Biotech) for 1 minute and immediate detection by exposure of membrane to an x-ray photographic film (MXB film, GRI autoradiography blue sensitive, Kodak) in a dark room using an exposure time of 1 minute. A representative gel is shown in Figure 2-23.

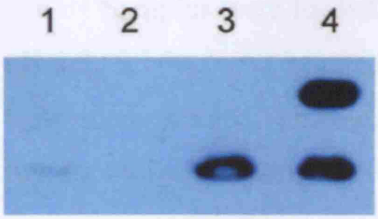


Figure 2-23 Western blot of refolded complex

This western blot shows the separated HLA heavy chain of a refolded HLA/peptide complex before biotinylation in lane 1, unbiotinylated MBP control in lane 2, the separated heavy chain of a refolded HLA/peptide complex post biotinylation in lane 3 and the same complex mixed with biotinylated MBP control in lane 4.

Figure 2-23 illustrates a faint signal from HLA heavy chain that was separated from the unbiotinylated HLA/peptide complex, which is likely to be due to minimal biotinylation occurring at the time of bacterial expression, as the BirA enzyme is constitutive of *E. coli* bacteria. In contrast, no signal was observed for the unbiotinylated MBP control, whereas both the biotinylated MBP control and the HLA heavy chain separated from the biotinylated HLA/peptide complex were detected as an upper (representing a molecular weight of MBP with Avitag of 44,846 kDa plus 244 Da of biotin) and a lower

band (representing a molecular weight of 34 kDa of the HLA heavy chain protein) respectively.

2-11.13 Estimation of biotinylation efficiency by native gel

Biotinylation efficiency was estimated using 2 µg biotinylated or unbiotinylated monomers, each with or without incubation with 3 µg or 6 µg ExtrAvidin (Sigma) for one hour at room temperature. All samples were run on an 8 % native gel consisting of a resolving lower gel (375 mM Tris pH 8.8, 27 % ProtoGel, 0.3 % (w/v) APS and 0.03 % (v/v) TEMED) overlaid with a stacking upper gel (125 mM Tris-HCl pH 6.8, 17 % ProtoGel (containing 30 % (w/v) Acrylamide and 0.8 % (w/v) Bis-Acrylamide; National Diagnostics), 1 % APS (w/v) and 0.1 % (v/v) TEMED) that had been pre-run at 150 volts for 30 minutes in 24.8 mM Tris/192 mM Glycin to remove any excess salts.

Unlike SDS-PAGE type electrophoreses (compare section 2-11.8), native gel electrophoresis does not use a charged denaturing agent. The proteins being separated are not denatured but remain in their native state, therefore differ in molecular mass and intrinsic charge and experience different electrophoretic forces dependent on their charge-to-mass ratio.

Samples were loaded with an equal volume of loading buffer (50 mM Tris-HCl pH 8.8, 0.1 % (w/v) bromphenol blue and 10 % (v/v) glycerol) and run at 150 volts. Gels were stained, photographed and dried as described in section 2-11.8 with a representative gel shown in Figure 2-24.

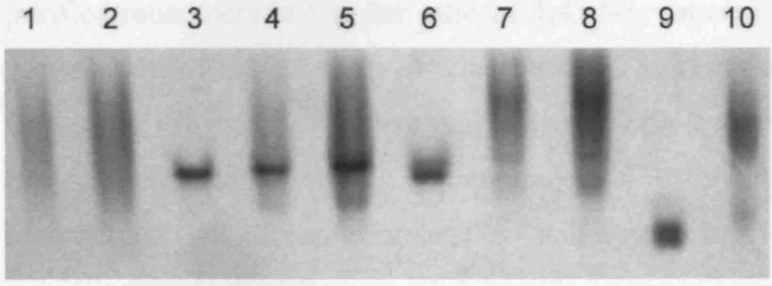


Figure 2-24 Shift of biotinylated proteins in native gel

This native gel demonstrates 3 µg and 6 µg of ExtrAvidin in lanes 1 and 2 respectively. Lanes 3-5 contain 2 µg unbiotinylated protein complex either alone (lane 3) or after incubation with 3 µg (lane 4) or 6 µg (lane 5) ExtrAvidin. Lanes 6-8 contain 2 µg biotinylated protein complex either alone (lane 6) or after incubation with 3 µg (lane 7) or 6 µg (lane 8) ExtrAvidin. Lanes 9-10 contain 1µg commercially biotinylated product BSI 300 (Avidity) either alone (lane 9) or after incubation with 3 µg ExtrAvidin (lane 10) as a positive control.

Native gels were used to estimate the efficiency of biotinylation of generated protein complexes. Successful biotinylation resulted in a shift of product when incubated with

biotin binding ExtrAvidin in contrast to product on its own (lanes 7 and 8 in comparison to lane 6) in native gels. This was due to biotinylated products forming a complex with ExtrAvidin and therefore running slower in the gel. The abundance of unshifted and shifted protein bands was used to estimate the percentage of monomer that was biotinylated efficiently. This estimate was taken into account for calculation of the amount of fluorescently labelled streptavidin to be added during tetramerisation of HLA/peptide complexes.

2-11.14 Tetramerisation of biotinylated monomers

Biotin binds very tightly to the tetrameric protein avidin. As an alternative 2-iminobiotin may be used, which is a cyclic guanidino analogue of biotin whose interaction with avidin is pH dependent and thus reversible (Orr, 1981).

Streptavidin is a homologue of avidin found in *Streptomyces avidinii.* It is one of the strongest noncovalent biological interaction known with a dissociation constant in the order of $4x10^{-14}$ M and can only be broken above 70 °C (Holmberg *et al.*, 2005). The same interaction occurs with ExtrAvidin that was used in reactions described earlier (sections 2-11.12 and 2-11.13), which lacks the "RGD" (Arginine-Glycine-Aspartic acid, an attachment site for adhesive extracellular matrix proteins such as integrins that can influence immunological functions (Ruoslahti, 1996)) sequence present in streptavidin.

PE or APC conjugated streptavidin (Biogenesis) was added to biotinylated purified monomers at a molar ratio of 1:4. This process was performed stepwise and very slowly within 3.5 hours shaking on ice. The resulting tetramers (in 10 mM Tris-HCl pH 8, 150 mM NaCl) were supplemented with 0.1 % (w/v) sodium azide and 10 % (v/v) glycerol and kept at -20 °C for storage or 4 °C for short-term use. The composition of tetrameric HLA/peptide molecules is illustrated in Figure 2-25. In comparison, a crystallographic model of an HLA/peptide tetramer complex can bee seen in Figure 1-12 on page 80.

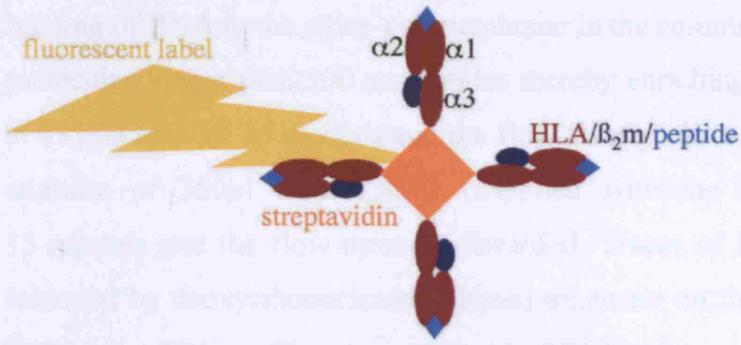


Figure 2-25 Composition of HLA/peptide tetramer

This figure illustrates the binding of four biotinylated HLA(●)/ß$_2$m(●)/peptide(◆) monomers to streptavidin (◆) conjugated to a fluorescent label (yellow).

2-12 Spectratyping

2-12.1 RNA extraction

Several RNA extraction methods were tested initially (described in section 4-2.1). The final method used for extraction of RNA from small numbers of tetramer sorted cells (<5,000) is described below. Alterations made during m-RNA extraction from larger cell numbers are described thereafter. To eliminate RNase and DNA contamination, experiments were performed in a fume hood dedicated to RNA work. Disposable filter tips were used and all labware such as the hood surface and pipettes were cleaned with "RNase AWAY" reagent (Invitrogen) before use.

PBMC were spun at 13,000 rpm for 1 minute and the supernatant aspirated. Cell pellets were used immediately or stored at -70 ˚C for RNA extraction at a later time. Therefore fresh or thawn pellets were kept on ice and total RNA was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions.

Samples were lysed and homogenised using guanidine-isothiocyanate lysis. 75 µl of buffer RLT (guanidine thiocyanate containing buffer provided with the kit) containing 1 % (v/v) ß-mercaptoethanol (BDH Biochemicals) was added to the cell pellet, and the sample was mixed by vortexing for 1 minute. After addition of 5 µl of a 4 ng/µl poly-A RNA carrier solution to the lysate, homogenisation was performed by resuspension using a fine pipette tip. This resulted in disruption of plasma membranes, release of RNA, shearing of high molecular weight genomic DNA and reduction of viscosity of the cell lysate. 75 µl of 70 % sterile, American Chemical Society (ACS) grade ethanol (Sigma Aldrich) was added to provide ideal binding conditions. The well-mixed lysate was then loaded onto a column sitting in a collection tube resulting in

binding of RNA to the silica-gel-membrane in the column. This procedure isolates RNA molecules longer than 200 nucleotides thereby enriching for mRNA. Tubes were spun at 13,000 rpm for 15 seconds and the flow-through discarded. Samples were washed by addition of 350µl buffer RW1 (supplied with the kit), spun at 13,000 rpm for 15 seconds and the flow-through discarded. Traces of DNA that may co-purify were removed by deoxyribonuclease (DNase) treatment on the column using digestion with RNase-free DNase. Therefore DNase I (2.72 Kunitz units/µl) was diluted 1:8 in buffer RDD (supplied with the kit) and 80 µl were pipetted directly onto the membrane for incubation at room temperature for 15 minutes. DNase and contaminants were then washed off by addition of 350 µl buffer RLT and centrifugation at 13,000 rpm for 15 seconds. The flow through was discarded and the column transferred to a new collection tube for a further wash with 500 µl buffer RPE (supplied with the kit). After centrifugation and discarding of the collection tube, 500 µl of 80 % sterile, ACS grade ethanol (Sigma Aldrich) were added and the membrane dried by centrifugation at 13,000 rpm for 2 minutes. The centrifugation was repeated in a new collection tube at 13,000 rpm for a further 5 minutes. Purified RNA was captured in a new collection tube by elution with 12 µl RNase-free water and centrifugation at 13,000 rpm for 1 minute resulting in a final volume of approximately 10 µl RNA. It was either used for RT-PCR immediately or stored at –70 °C.

When using cell numbers above 5,000, the same method was used. However, no poly-A RNA carrier was used and the volume of buffer RLT added to cell pellets and that of 70 % ethanol added to the lysate was 350 µl instead of 75 µl. If cell pellets were large enough to be visible, homogenisation was performed by passing of the lysate through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe instead of vortexing and resuspension.

2-12.2 Reverse transcription PCR

Most reverse transcriptases are intended for use with approximately 1 µg RNA. For reverse transcription (RT) PCR of RNA obtained from small cell numbers, optical absorbance measurement to establish the concentration of RNA solution obtained by RNA extraction was omitted. This was to avoid loss of material and because carrier RNA was used during the extraction of RNA. The total of extracted RNA was used for RT PCR with sensiscript reverse transcriptase (Qiagen), an enzyme designed for RT PCR of small amounts of RNA. Therefore the total volume of RNA (10 µl) was denatured in the presence of 1 µl of 500 µg/µl random primers (Promega) and 2 µl of

5 mM dNTP (provided with the sensiscript reverse transcriptase) at 65 °C for 5 minutes and subsequently chilled on ice immediately before the reaction. 7 µl of master mix (containing 2 µl of 10X reaction buffer (provided with the sensiscript reverse transcriptase), 1 µl of recombinant RNasin ribonuclease inhibitor (Promega), 1 µl of sensiscript reverse transcriptase (Qiagen) and 3 µl of RNase free water (provided with the sensiscript reverse transcriptase) per sample) was then added to the sample for incubation at 37 °C for 1 hour. During this reaction sensiscript reverse transcriptase transcribes complementary DNA from the RNA template (RNA-dependent DNA-polymerase activity) and degrades RNA in RNA:DNA hybrids (RNase H activity). This resulted in a total volume of 20 µl cDNA for immediate use in PCR reactions or storage at -20 °C.

RT-PCR from high numbers of cells (not post FACS sort) was initially performed using 1 µg RNA solution in a volume of 10 µl with 1 µl of 500 ng/µl random primers (Promega) and 1 µl of 10 mM dNTPs (Bioline) for 5 minutes at 65 °C and subsequent chilling on ice. The resulting 12 µl template was reverse transcribed in a 20 µl total reaction volume with 400 units Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Promega), 10 mM DTT (Sigma) and 80 units RNasin RNase inhibitor (Promega) in M-MLV RT buffer (Promega) by incubation at room temperature for 10 minutes followed by 50 minutes at 37 °C. The reaction was terminated by incubation at 70 °C for 15 minutes and the resulting cDNA was stored at -20 °C. During optimisation procedures, RNA extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol resulted in an elution volume of 20 µl and was subsequently used for M-MLV reverse transcription using double the volumes mentioned above.

2-12.3 Confirmation of c-DNA quality by ß-Actin PCR amplification

0.5 µl c-DNA was amplified in a 25 µl reaction volume using 0.5 units Taq (Bioline), 2 mM $MgCl_2$ (Bioline), 0.4 mM dNTPs (Bioline), 0.4 mM 5'ß-actin primer with the sequence 5' TCA TGA AGT GTG ACG TTG ACA TCC GT 3' (Promega) and 0.4 mM 3'ß-actin primer with the sequence 5' CTT AGA AGC ATT TGC GGT GCA CGA 3' (Promega) in PCR buffer (Bioline).

PCR conditions were 94 °C for 2 minutes followed by 94 °C for 30 seconds, 65 °C for 1 minute and 68 °C for 2 minutes for 40 cycles and final polymerisation at 68 °C for 7 minutes.

5 µl of PCR products were subjected to gel electrophoresis as described in section 2-8.5 to test amplification of the constitutive gene ß-actin.

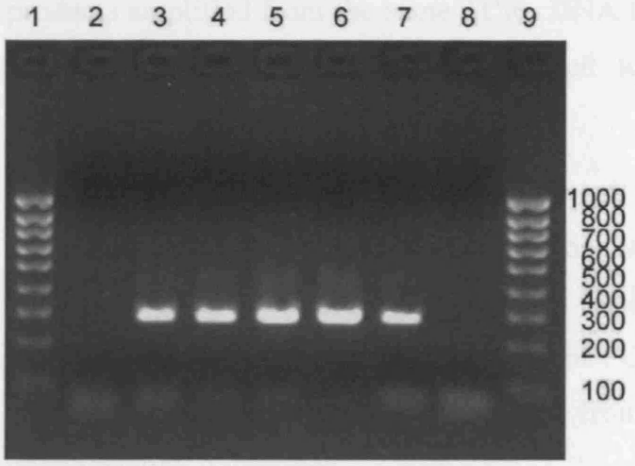


Figure 2-26 ß-Actin PCR

This figure shows separation of 15 µl of ß-Actin PCR products (285 bp) on a 1.5 % agarose (0.5x) TBE gel that was run at 100 volts. Shown are PCR products obtained from cDNA from 188 (lane 2, not visible), 375 (lane 3), 750 (lane 4), 1,500 (lane 5), 3,000 (lane 6) (174xCEM) T2 cells along with a positive control of cDNA obtained from 300,000 PBMC (lane 7) and a negative control of non-template RT PCR product (lane 8) and Hyperladder IV (Bioline) in lane 1 and 9. The molecular weight of the marker's standards (in bp) is shown beside the gel picture.

2-12.4 Fluorescent TCR Vß multiplex PCR

1 µl c-DNA was amplified in a 12.5 µl reaction volume using 0.265 units Platinum *Taq* (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.4 mM dNTPs (Bioline), 0.4 µM unlabelled constant region Cß primer and a mixture of labelled Vß primers at optimised concentration ratios (Table 2-7) in PCR buffer (Invitrogen). PCR conditions were 95 °C for 1 minute followed by denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds and primer extension at 72 °C for 5 minutes for 30 cycles followed by a final polymerisation step at 72 °C for 10 minutes.

Platinum *Taq* DNA polymerase was chosen for this application because it is complexed with a proprietary antibody that inhibits polymerase activity until it is denatures at 94 °C. This results in a number of advantageous properties including reduced primer dimer formation.

Initial optimisation experiments demonstrated superior amplification results with an extended period of the primer extension step at 72 °C for 5 minutes. The experiments were initially performed to test whether long PCR extension periods may result a more uniform detection of PCR product lengths during spectratyping. *Taq* DNA polymerase has a nontemplate-dependent terminal transferase activity that adds a single

deoxyadenosine (A) to the 3' ends of some of the PCR products. Therefore spectratyping may demonstrate 2 peaks with a size difference of one nucleotide in PCR products amplified from the same TCR cDNA template. Long extension cycles did not influence this phenomenon but continued to be used because they gave better amplification results.

7 multiplex PCR reactions (A-G) were performed per sample. These included optimised ratios of primer concentrations, which were multiplexed according to compatibility and amplicon size as listed in Table 2-7. Ratios of primers were optimised to achieve similar fluorescence intensities of PCR products from all Vß families within each PCR reaction from c-DNA obtained from total PBMC from CMV seronegative young healthy volunteers.

Mix	Molarity	Specificity	5' label	Primer sequence	Amplicon size
A	2.36 µM	Vß1 primer	FAM	5-CAA CAG TTC CCT GAC TTG CAC-3	185–214 bp
	1.59 µM	Vß2 primer	JOE	5-TCA ACC ATG CAA GCC TGA CCT-3	182–220 bp
	3.19 µM	Vß5S2 primer	TAMRA	5-CCT AAC TAT AGC TCT GAG CTG-3	176–214 bp
	13.29 µM	Vß5S1 primer	JOE	5-ATA CTT CAG TGA GAC ACA GAG AAA C-3	242-271 bp
B	2.66 µM	Vß6 primer	FAM	5-AGG CCT GAG GGA TCC GTC TC-3	182–211 bp
	2.66 µM	Vß7 primer	JOE	5-CTG AAT GCC CCA ACA GCT CTC-3	188–220 bp
	3.99 µM	Vß8 primer	FAM	5-TAC TTT AAC AAC AAC GTT CCG-3	248–277 bp
	13.29 µM	Vß9 primer	TAMRA	5-AAA TCT CCA GAC AAA GCT CAC-3	185–217 bp
	31.88 µM	Vß16 primer	JOE	5-GAG TCT AAA CAG GAT GAG TCC-3	229–261 bp
C	3.99 µM	Vß12 primer	FAM	5-GAC AAA GGA GAA GTC TCA GAT-3	219–248 bp
	2.66 µM	Vß13S2 primer	TAMRA	5-GTT GGT GAG GGT ACA ACT GCC-3	236–271 bp
	1.99 µM	Vß13S1 primer	JOE	5-GAC CAA GGA GAA GTC CCC AAT-3	218–247 bp
D	2.39 µM	Vß11 primer	FAM	5-ACA GTC TCC AGA ATA AGG ACG-3	186–218 bp
	2.92 µM	Vß20 primer	TAMRA	5-TCT GAG GTG CCC CAG AAT CTC-3	212–244 bp
	26.57 µM	Vß15 primer	JOE	5-GTC TCT CGA CAG GCA CAG GCT-3	188–214 bp
E	1.33 µM	Vß3 primer	FAM	5-TCT AGA GAG AAG AAG GAG CGC-3	185–214 bp
	10.63 µM	Vß24 primer	TAMRA	5-AAA GAT TTT AAC AAT GAA GCA GAC-3	232–261 bp
	5.31 µM	Vß17 primer	JOE	5-CAC AGA TAG TAA ATG ACT TTC AG-3	235–270 bp
F	2.66 µM	Vß18 primer	FAM	5-GAG TCA GGA ATG CCA AAG GAA-3	220–246 bp
	15.94 µM	Vß23 primer	TAMRA	5-TCA TTT CGT TTT ATG AAA AGA TGC-3	248–277 bp
	16.63 µM	Vß14 primer	FAM	5-TCT CGA AAA GAG AAG AGG AAT-3	185–211 bp
	6.64 µM	Vß21 primer	FAM	5-GAT ATG AGA ATG AGG AAG CAG-3	242–280 bp
G	2.6 µM	Vß4 primer	FAM	5-CAT ATG AGA GTG GAT TTG TCA TT-3	222–251 bp
	7.97 µM	Vß22 primer	TAMRA	5-CAG AGA AGT CTG AAA TAT TCG A-3	221–250 bp

Table 2-7 Vß primer multiplexing

This table demonstrates the sequences and labels of Vß family specific primers and the molarity at which they were used in multiplex PCR reactions with Cß primer 5' CTT CTG ATG GCT CAA ACA C 3' to obtain amplicons of the range of size listed.

All Vß primers were labelled at the 5' end and were purchased from Sigma Genosys except Vß21, which was purchased from ABI. The Cß primer was unlabelled and was of the sequence 5' CTT CTG ATG GCT CAA ACA C 3' (Alta Biosciences). JOE (6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein) labelled primers were of HPLC grade. Due to limited resources FAM and TAMRA labelled primers were not HPLC purified. Lack of HPLC purification posed no challenge for FAM labelled primers. Singleplex PCR using unlabelled Vß primers and FAM labelled Cß primer were performed to confirm results for amplifications from Vß families Vß13S2, 22 and 24 because initial test experiments demonstrated that free TAMRA dye runs within the size area of interest for these amplifications.

Primer sequences used were in accordance with Fernandes and colleagues (Fernandes *et al.*, 2005) except for Vß5S1. Labels used and multiplexing of primers were modified from this report. The sequence of the Vß5S1 primer (Choi *et al.*, 1989) was chosen due to superior amplification obtained with this primer than with Vß5S1 primer used by Fernandes and colleagues, which lacks the first 4 nucleotides at its 5' end.

To ensure uniformity, Vß multiplex PCR was performed by preparation of a primer master mix for each of the 7 reactions and a master mix containing the other PCR reagents. The latter was divided into smaller master mixes that contained cDNA from one sample each and which in turn were combined with the appropriate amount of one of the seven primer master mixes before PCR amplification.

Initial primer sequences used during optimisation of the Vß PCR are shown in Table 2-8.

Label	Specificity	Primer sequence (5'-3')
none	Vß1	GCACAACAGTTCCCTGACTTGCAC
none	Vß2	TCATCAACCATGCAAGCCTGACCT
none	Vß3	GTCTCTAGAGAGAAGAAGGAGCGC
none	Vß4	ACATATGAGAGTGGATTTGTCATT
none	Vß5.1	ATACTTCAGTGAGACACAGAGAAAC
none	Vß5.2-3	TTCCCTAACTATAGCTCTGAGCTG
none	Vß6	AGGCCTGAGGGATCCGTCTC
none	Vß7	CCTGAATGCCCCAACAGCTCTC
none	Vß8	CCATCATGCGGGGACTGGAGTTGC
none	Vß9	CCTAAATCTCCAGACAAAGCT
none	Vß10	CTCCAAAAACTCATCCTGTACCTT
none	Vß11	TCAACAGTCTCCAGAATAAGGACG
none	Vß12	AAAGGAGAAGTCTCAGAT
none	Vß13.1	CAAGGAGAAGTCCCCAAT
none	Vß13.2	GGTGAGGGTACAACTGCC
none	Vß14	GTCTCTCGAAAAGAGAAGAGGAAT
none	Vß15	AGTGTCTCTCGACAGGCACAG
none	Vß16	AAAGAGTCTAAACAGGATGAGT
none	Vß17	CAGATAGTAAATGACTTTCAG
none	Vß18	GATGAGTCAGGAATGCCAAAGGAA
none	Vß19	CAATGCCCCAAGAACGCACCCTG
none	Vß20	ACGTCTGAGGTGCCCCAGAAT
none	Vß21	ATTCACAGTTCGGTAAGGATCGA
none	Vß22	GGGCAGAAACTCGAGTTTCTGGTT
none	Vß23	TTTATGAAAAGATGCAGAGCGAT
none	Vß24	AAGTCAAGTCAGGCCCCAAAGCT
FAM	Cß	CACCCACGAGCTAGCTCCACGTGGTC

Table 2-8 Vß primers used for singleplex PCR

This table demonstrates the sequences and labels of Vß family and Cß specific primers (Alta Biosciences) that were used at 0.4 mM in initial single PCR reactions.

Initial singleplex Vß PCR amplifications were performed using 1 µl c-DNA in a 25 µl reaction volume with 0.5 units Platinum *Taq* (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.4 mM dNTPs (Bioline), 0.4 mM FAM labelled constant region primer and 0.4 mM unlabelled Vß primer (Table 2-8) in PCR buffer (Invitrogen). PCR conditions were 95 °C for 1 minute followed by denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds and primer extension at 72 °C for 1 minutes for 29 cycles followed by a final polymerisation step at 72 °C for 10 minutes.

2-12.5 Fragment analysis by capillary gel electrophoresis

2 µl of 1:10 dilutions of PCR products were mixed with 7.75 µl 0.1 % (v/v) Tween 20 (BDH Biochemicals) and 0.25 µl energy transfer (ET)-ROX (5-carboxy-X-rhodamine) labelled size standard (ET-ROX 550, Amersham Biosciences) containing 22 bands at

25 base pair periodicity ranging from 75 to 550 bp and two feature bands at 60 and 310 bp.

Determination of size and fluorescence intensity of PCR products was then performed with the linear polyacrylamide (LPA) separation polymer MegaBACE Long Read Matrix (Amersham Biosciences) on a MegaBACE 1000 (Amersham Biosciences), a fluorescence-based DNA analyser using an array of 96 capillaries for capillary gel electrophoresis. Therefore samples were pipetted into 96-well plates (Robbins Scientific) and denatured at 95 °C for 5 minutes followed by chilling on ice. The sample plate, two control plates containing LPA buffer (Amersham Biosciences) and 6 vials containing MegaBACE Long Read Matrix (Amersham Biosciences) were spun at 10,000 rpm for 2 minutes before loading into the MegaBACE instrument. The settings used were 3 kV injection voltage, 10 kV run voltage, 60 seconds injection time and 80 minutes run time at 44 °C.

Traditional gel electrophoresis can separate DNA fragments by using an electric current applied to a gel matrix. DNA migrates from negative (cathode) to positive (anode) electrodes due to the naturally occurring negative charge carried by their sugar-phosphate backbone (compare section 2-8.5).

During capillary electrophoresis in the MegaBACE, an electric field is used to cause the DNA fragments to migrate into (electrokinetic injection) and through (electrophoresis) the capillaries. As in gel electrophoresis, the DNA fragments are separated by size, with the shorter fragments moving faster (early peaks in the electropherogram) than the longer fragments (late peaks in the electropherogram). The large surface-to-volume ratio of the capillary efficiently removes the heat generated during electrophoresis. With less heat, capillary electrophoresis can be performed at a higher voltage, which produces more rapid fragment migration and shorter separation times. The DNA fragments are detected near the outlet end of the capillary. The output of the detector is sent to a computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated products appear as peaks with different retention times in an electropherogram.

To record four dyes separately, the MegaBACE 1000 instrument uses an emission optical pathway consisting of beamsplitters, filters, and photomultiplier tubes (PMTs) that detect laser induced fluorescence. Light from two lasers is used to scan the capillaries containing the fluorescently labelled samples. It excites the fluorescent dyes, which then emit fluorescent light. Two beamsplitters are used to split the emitted

fluorescent light. Each of four filters permits only a specific range of light to pass through, which corresponds to the emissions of one of the dyes. PMTs are then used to detect the filtered light and convert the light into electrical current, producing an electropherogram for each capillary. A blue laser (488 nm) is used during a forward scan of samples whereas a green laser (532 nm) is used during the return scan.

Figure 2-27 illustrates detection of size standards from the ET-ROX ladder in channel 1 from emitted light through beamfilter A and filter 1 and detection of PCR products amplified with unlabelled Cß and FAM labelled Vß primers in channel 2 from emitted light through beamfilter A and filter 2. Fluorescence from the ladder and the described PCR products is induced by the 488 nm laser during the forward scan of capillaries. The beamsplitter has a wavelength cut off of 540 nm, thereby allowing light emitted by ET-ROX (maximum 607 nm) to pass whereas light emitted by FAM (maximum 517 nm) is reflected. The figure further illustrates detection of PCR products amplified with unlabelled Cß and TAMRA (N,N,N',N'-tetramethyl-5-carboxyrhodamine) labelled Vß primers in channel 3 from emitted light through beamfilter B and filter 3, and detection of PCR products amplified with unlabelled Cß and JOE labelled Vß primers in channel 4 from emitted light through beamfilter B and filter 4. Fluorescence from both types of PCR products is induced by the 532 nm laser during the return scan of capillaries. The beamsplitter has a wavelength cut off of 570 nm, thereby allowing light emitted by TAMRA (maximum 574 nm) to pass whereas light emitted by JOE (maximum 548 nm) is reflected.

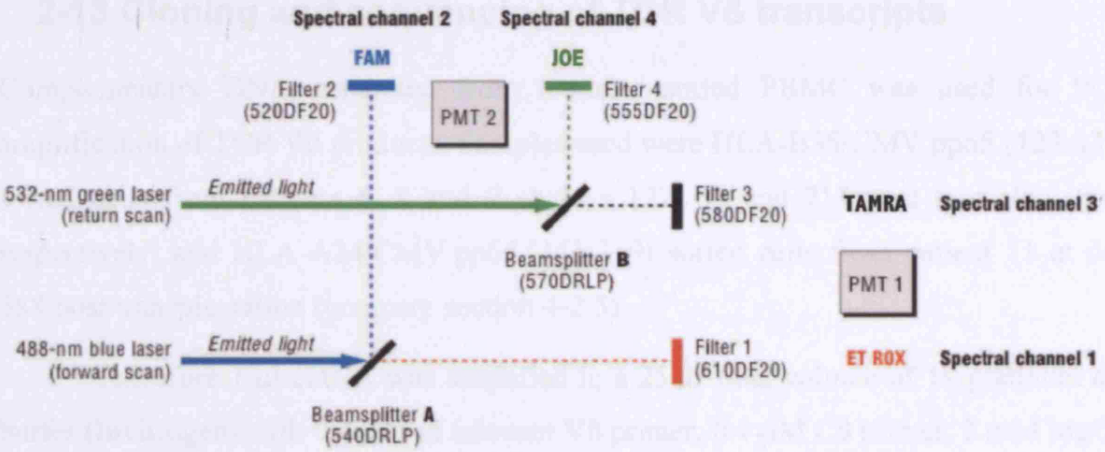


Figure 2-27 The MegaBACE's emission optical pathway for the detection of laser induced fluorescence

This figure is modified from an illustration from the MegaBACE 1000 manual (Amersham Biosciences). It demonstrates the set of filters and beamsplitters used during capillary gel electrophoresis for the detection of emission of dyes (that were used for TCR Vß PCR) in four different channels in the MegaBACE instrument.

The ET-ROX size standard uses energy transfer. Two fluorescent dyes are attached to the same molecule. FAM acts as a donor dye and ROX as an acceptor dye. The blue laser emits light at 488 nm, which is near the absorption maximum of FAM. FAM absorbs the blue laser light and then transfers the energy to excite ROX, which then emits its characteristic fluorescent light spectrum. Energy transfer results in the effectively emitted signal of dual labelled molecules to be greatly increased compared to the signal from molecules labelled with single dyes (Ju *et al.*, 1996).

During fluorescence detection on the MegaBACE, spectral overlap can occur as described in section 2-5.3 (compare Figure 2-4, page 110). The PMT converts light energy into an electrical current. Increasing the voltage applied to a PMT increases the signal amplification. The PMT voltages used for optimal recording of the dyes were selected after a spectral overlay matrix was generated. Therefore 2 µl of each of the FL, JOE and TMR standards from the matrix standard reagent "Matrix FL-JOE-TMR-CXR" (Promega) and 0.25 µl ET-ROX standard were each loaded in duplicate with 8 µl 0.1 % (v/v) Tween 20, denatured and run as described above. The resulting file was then used to create a spectral overlap matrix according to the manufacturer's instructions. Data was then analysed with Genetic Profiler software Version 1.1 (Amersham Biosciences).

2-13 Cloning and sequencing of TCR Vß transcripts

Complementary DNA generated from tetramer sorted PBMC was used for PCR amplification of TCR Vß products. Samples used were HLA-B35/CMV pp65 (123-131) sorted cells from patients 4, 8 and 9 at days 122, 53 and 713 post transplantation, respectively, and HLA-A24/CMV pp65 (341-349) sorted cells from patient 11 at day 588 post transplantation (compare section 4-2.5).

Therefore 1 µl cDNA was amplified in a 25 µl final volume of 1x platinum *taq* buffer (Invitrogen) with 0.4 µM of relevant Vß primer, 0.4 µM Cß primer, 2 mM $MgCl_2$ (Invitrogen), 0.4 mM dNTPs (Bioline) and 0.265 units platinum *taq* (Invitrogen) at 95 °C for 1 minute followed by denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds and primer extension at 72 °C for 5 minutes for 30 cycles followed by a final polymerisation step of 10 minutes at 72 °C.

cDNA from HLA-B35/CMV pp65 (123-131) sorted cells was amplified with Vß primers 6 and 12 whereas cDNA from HLA-A24/CMV pp65 (341-349) sorted cells was amplified with Vß primers 7 and 20. The sequences of primers were as listed in Table 2-7 but without fluorescent labels.

PCR products were run on a 1.5 % agarose TBE gel and products of the expected size range between 182 and 248 bp (compare Table 2-7) were purified using a GFX purification kit as described in section 2-8.6. 4 µl of purified DNA solution was ligated into pCR2.1 for transformation of TOP10F' cells and plasmid DNA extraction from resulting bacterial clones as described in section 2-11.1. GFX cleaned DNA from clones that demonstrated presence of inserts by EcoRI digest testing was used for sequencing (Advanced Biotechnology Centre, Imperial College, London, UK) using M13 forward (5′-GTA AAA CGA CGG CCA G-3′) and M13 reverse (5′-CAG GAA ACA GCT ATG AC-3′) primers provided with the TOPO-TA cloning kit (Invitrogen). Sequencing was performed using BigDye V3.1 chemistry on an Applied Biosystems 3100 capillary sequencer using 96 °C 10 seconds, 46 °C 5 seconds, 60 °C 4 minutes, 35 cycles and results were analysed using IMGT/V-QUEST.

2-14 Data collection and analysis

Collected data was analysed using Microsoft Office 2004 for Macintosh, Kaleidagraph Version 4.03 (Synergy), EditView Version 1.0.1, CLC DNA Workbench Version 3 or instrument specific software such as Genetic Profiler Version 1.1 (Amersham Biosciences) for analysis of files generated on the MegaBACE 1000 DNA sequencer and ABI 7500 SDS software Version 1.2 (Applied Biosystems) for analysis of data generated by quantitative PCR. For analysis of TCR sequences the IMGT/V-QUEST web tool (http://imgt.cines.fr) was utilized and for analysis of flow cytometry data FlowJo Version 6.0 software (Tristar) was used. Chemical formula drawing was performed with ChemDraw Ultra Version 11.0.1 (CambridgeSoft).

Statistical tests were carried out using the Prism 3 software (GraphPad) package using a two-tailed Mann-Whitney U test.

CHAPTER 3 ESTABLISHING PROTECTIVE LEVELS OF CD8$^+$ T CELLS WITH DIFFERENT HLA/CMV PEPTIDE TARGETS IN HSCT PATIENTS

3-1 Introduction

Following primary infection CMV causes mild or subclinical illness in immunocompetent hosts and enters a latent phase that usually persists for the life of the host. The first chapter introduced the importance of CMV reactivation in profoundly immunosuppressed HSCT patients. CMV infection occurs in 69 % of CMV seropositive patients (Meyers *et al.*, 1986) and remains a significant cause of morbidity and mortality. CMV specific CD8$^+$ T cells are thought to be required to maintain the virus in a latent state and investigations during this project aim to further the knowledge of the role of these cells in patients.

Due to the limitations of current antiviral therapies, alternative approaches, such as an adoptive transfer of donor-derived CMV specific CD8$^+$ T cell clones, are of great interest in the field. It was demonstrated that CMV specific CD8$^+$ T cells inversely correlate with viral replication and clinical status in patients (Aubert *et al.*, 2001, Cwynarski *et al.*, 2001). Adoptive cell transfer has been shown to reduce the rate of viral reactivation and may therefore combat potentially fatal CMV related complications in HSCT patients (Einsele *et al.*, 2002, Walter *et al.*, 1995). To make this therapy available to a wide range of patients expressing different HLA alleles, it is important to address the question of which number of CMV specific CD8$^+$ T cells restricted by a variety of HLA alleles may inversely correlate with the ability to detect CMV reactivation in patients.

Previous studies mainly focused on enumeration of CMV specific CD8$^+$ T cell responses restricted by HLA-A2 and HLA-B7 (Aubert *et al.*, 2001, Cwynarski *et al.*, 2001, Engstrand *et al.*, 2000, Gillespie *et al.*, 2000, Gratama *et al.*, 2001, Komatsu *et al.*, 2003, Mohty *et al.*, 2004, Ozdemir *et al.*, 2002, Singhal *et al.*, 2000). It was found that recovery of $1x10^7$ (Cwynarski *et al.*, 2001) to $2x10^7$ (Aubert *et al.*, 2001) HLA-A2/pp65 specific CD8$^+$ T cells/L of blood is associated with protection from CMV disease in patients.

Currently, however, it is unknown at what level CMV specific CD8$^+$ T cells targeting other common HLA/peptide combinations may be correlating with protection

against CMV. This study investigates $CD8^+$ T cells towards multiple CMV peptides that were previously shown to be presented by common HLA alleles (compare Chapter 1). Their "protective levels" may be regarded as surrogate markers for the total CMV specific response that correlates with protection against CMV in HSCT patients, which will be discussed in section 3-3.2.

This chapter describes the pattern of recovery of CMV specific $CD8^+$ T cells targeting different HLA/peptide combinations and the correlation of these cells with protection from CMV in HSCT patients. Section 3-2 describes the longitudinal monitoring of individual patients followed by statistical calculations of cell numbers that inversely correlated with the ability to detect CMV reactivation in four subsections depending on the different HLA/peptide combinations studied. It demonstrates a detailed qualitative and quantitative analysis of individual patient responses highlighting interesting observations and grouping patient responses according to occurrence of CMV reactivation and detection of CMV specific $CD8^+$ T cells.

Section 3-3 demonstrates the main findings from the quantitative analysis of CMV specific $CD8^+$ T cells targeting different HLA/peptide combinations and serves to integrate and compare all major findings from section 3-2. It also contains a comparison with recent literature, conclusions and implications of results.

Immune responses were investigated using sensitive HLA/peptide tetramers. The generation of several different reagents for this purpose was a main part of this project. The results obtained during optimisation of the production of the tetramers is described in Chapter 2, section 2-11 rather than in this chapter to draw the reader's attention to the outcome of T cell measurements in this chapter. These measurements were correlated to CMV DNAemia, measured by quantitative PCR during routine viral monitoring by the Virology department, Royal Free Hospital for all patients except patients 10 and 15. In patient 10 CMV load was measured at the Universidad Autónoma de Madrid in Spain. In patient 15 CMV was detected by antigenaemia rather than DNAemia using perinuclear staining of leukocytes indicating the number of CMV infected cells per 200,000 PBMC (van der Bij *et al.*, 1988) stained during routine viral monitoring by the Virology department of the Universidade de Federal do Paraná in Brazil. Measurements of tetramer binding cells and CMV load were performed on peripheral blood of patients taken at frequent time points post transplantation.

The functionality of T cells was assessed by ELISpot assays testing the release of INFγ from cells upon stimulation with their target peptide. Besides CMV viral load

and CMV-specific $CD8^+$ T cell immunodeficiency, $CD4^+$ lymphopenia was also reported to be a predictor of late CMV disease and death after allogeneic stem cell transplantation (Boeckh *et al.*, 2003). Therefore total numbers of $CD4^+$ T helper cell counts, known to influence the functionality of $CD8^+$ T cells, were also measured and are correlated with the analysis of CMV specific $CD8^+$ T cell responses in this study.

HLA-A*0201 restricted $CD8^+$ T cell responses to CMV pp65 (495-503) are the most studied and best known cytotoxic T cell responses to CMV. Initial studies on CMV specific $CD8^+$ T cells focused on frequency measurements of $CD8^+$ T cells restricted by HLA-A*0201 because this allele is amongst the most common HLA type in Caucasoids and many other ethnic groups. Frequency measurements of CMV specific $CD8^+$ T cells restricted by HLA-A*0201 were therefore used as an archetype for comparison with responses targeting other HLA/peptide combinations in this study.

Prolonged antiviral treatment and the development of chronic graft *versus* host (GvH) disease are risk factors for the development of late-onset CMV disease in HSCT patients (Einsele *et al.*, 2000). A placebo-controlled study of ganciclovir treatment demonstrated that the suppression of viraemia by prolonged antiviral treatment results in a decrease in priming of CMV specific $CD8^+$ T cells (Li *et al.*, 1994). GvH disease (GvHD) necessitates immunosuppressive treatment, which impairs the development and function of CMV specific $CD8^+$ T cell responses. Most of the patients that received allogeneic HSCT in the study described here received aciclovir as antiviral prophylaxis and cyclosporine A alone or with methotrexate as GvHD prophylaxis (compare Table 2-1 on page 99). Illustrations demonstrating the pattern of recovery of CMV specific $CD8^+$ T cell responses in HSCT patients in this chapter indicate periods of active immunosuppression and antiviral treatment (usually intravenous ganciclovir at 5 mg/kg of body weight twice a day, intravenous foscarnet at 90 mg/kg of body weight twice a day or oral valganciclovir at 900 mg/kg of body weight twice a day, compare section 2-2.5) that was received by patients in addition to these prophylactic measurements.

It is appreciated that CMV specific $CD8^+$ T cell levels and CMV disease are also influenced by different transplant related factors (Peggs, 2004b). These include CMV serostatus and donor relationship to recipient (Cwynarski *et al.*, 2001, Ljungman *et al.*, 2003), conditioning regimens (Junghanss *et al.*, 2002), use of TCD (Chalandon *et al.*, 2006), type of transplant (Trenschel *et al.*, 2000), GvHD (Meyers *et al.*, 1986) and age of recipient (Enright *et al.*, 1993) or donor (Baron *et al.*, 2006). These parameters are therefore listed in Table 2-1 on page 99 specifying patient characteristics in Chapter 2.

3-2 Results

The outcome of the investigation of CMV specific $CD8^+$ T cell responses in HSCT patients is described below. Responses targeting different CMV peptides presented by different HLA alleles have been studied. As described in the introductory chapter (section 1-11), the HLA alleles chosen for analysis are HLA-A1, HLA-A2, HLA-A24 and HLA-B35. The pattern of HLA-B35 restricted responses is simpler to explain than the pattern of responses restricted by the other HLA types. This is because responses restricted by HLA-B35 were found to be more consistent amongst patients whereas individual circumstances resulted in responses restricted by other HLA types to be less consistent amongst patients. Therefore not all of the responses restricted by HLA types other than HLA-B35 could be considered for statistical analysis in the same way as responses restricted by HLA-B35. Responses restricted by HLA-B35 are therefore described first.

3-2.1 CMV specific $CD8^+$ T cell responses restricted by HLA-B35

This section will describe the observation that low numbers of CMV specific $CD8^+$ T cell responses restricted by HLA-B35 inversely correlated with the ability to detect CMV reactivation in HSCT patients. The CMV peptide target that was studied will be described first. Then the results of the longitudinal monitoring of patients will be shown. One sub section will be dedicated to responses observed in patient 7, which were suspected to be crossreactive and were investigated in more detail. A statistical comparison of $CD8^+$ T cells assigned to different groups depending on viral load measurements at the time of sampling will then be shown and response levels in healthy volunteers will be listed. A comparison to HLA-A*0201 restricted CMV pp65 (495-503) specific responses will be shown in a later section (section 3-3, from page 238).

3-2.1.1 CMV targets of $CD8^+$ T cell responses restricted by HLA-B35

As outlined in Chapter 1 (section 1-11.2), the CMV tegument phosphoprotein, pp65, is considered one of the most important antigens for cellular immunity in CMV infection.

Common subtypes of HLA-B35 demonstrate amino acid substitutions within the peptide binding cleft region. This micropolymorphism was reported to influence the relative binding stability and thereby immunodominance of viral peptides including pp65 between individuals expressing different HLA-B35 subtypes (Burrows *et al.*, 2007, Green *et al.*, 2004, Tynan *et al.*, 2005).

Responses restricted by the most common subtype, HLA-B*3501, are analysed in this section. These $CD8^+$ T cell responses target the 9mer located at the region pp65 123-131 with the sequence IPSINVHHY, which was described by Gavin and colleagues (Gavin *et al.*, 1993). Only one other CMV antigen (Kern *et al.*, 2002) of HLA-B*3501 restricted $CD8^+$ T cells had been reported at the time of initiation of this project. This epitope (pp65(173-183)) was reported in only one individual. In contrast pp65 (123-131) is thought to be the strongest known epitope in the context of HLA-B*3501 (Burrows *et al.*, 2007). Other $CD8^+$ T cell antigens derived from pp65, lie within the regions 181-195 and 187-201 overlapping at 187 to 195 (Wills *et al.*, 1996), of which peptide 187-195 was later reported to be restricted by HLA-B*3503 (Wills *et al.*, 2002b) whereas pp65 188-195 was reported to be restricted by HLA-B*3502 (Villacres *et al.*, 2003) and most recently HLA-B*3508 (Burrows *et al.*, 2007).

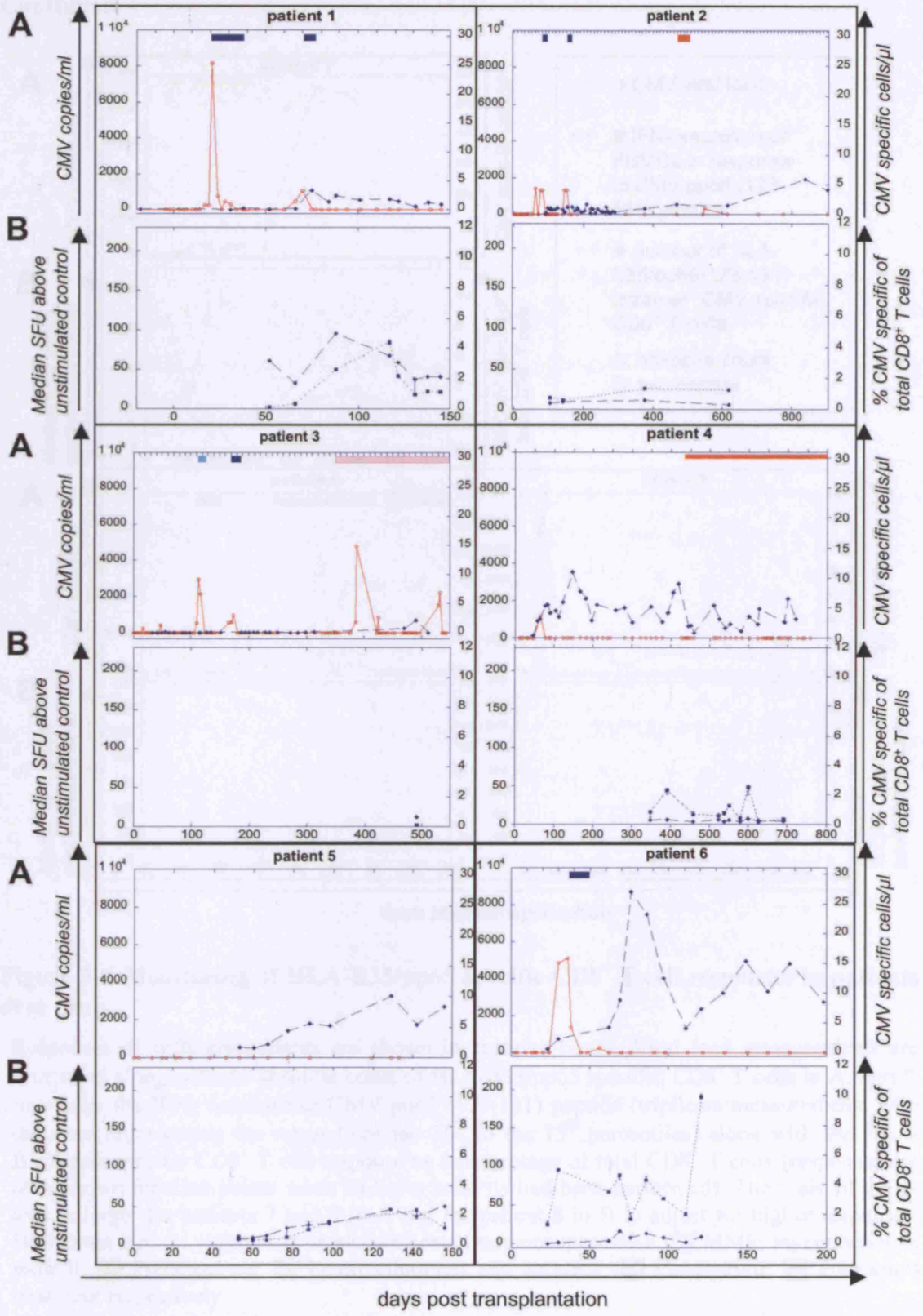
HLA-B*3501 restricted CMV pp65 (123-131) specific $CD8^+$ T cell responses investigated in this study will subsequently be abbreviated as HLA-B35/pp65 specific $CD8^+$ T cell responses.

3-2.1.2 Monitoring of CMV specific $CD8^+$ T cells in patients expressing HLA-B35

15 HSCT patients were originally recruited for the study of HLA-B35/pp65 specific $CD8^+$ T cell responses. They and their donors express HLA-B*3501; patient, donor, or both are CMV seropositive. Patients from whom samples were collected from earlier than 100 days post transplantation onwards with at least 4 collections post transplantation were included in the follow-up cohort (n = 9) to establish a threshold of protective levels of responses to this HLA/peptide target. Detailed patient characteristics are given in Table 2-1 on page 99. HLA-B35/pp65 specific $CD8^+$ T cells detected in the 9 patients are shown in this chapter. The average start of follow- up was day 44 (range day 23 - 88) and the average end of follow-up was day 481 (range day 60 - 902) post transplantation with an average of 19 samples taken per patient (range 4 - 37).

The pattern of recovery of CMV specific $CD8^+$ T cells observed in patients post HSCT is illustrated in line graphs. To best illustrate the pattern of responses seen in patients over time, samples were collected as frequently as once a week whenever possible. It must be noted, however, that the larger the gap between two samples taken (depending on patient's availability) the more likely it was that an intermittent peak or transient decrease of the response may have been missed and lines shown between data

points on the graph are used for easier viewing rather than assuming that they reflect the actual pattern of the response between data points measured. Tetramer binding cell numbers were correlated with viral load and functionality of cells was tested by IFNγ assays for samples with sufficient cell numbers. The results are summarised in Figure 3-1. Due to low cell numbers obtained from patient samples, ELISpot measurements in patient 3 and patient 8 were performed at a single time point only.


A
B
patient 1
patient 2
patient 3
patient 4
patient 5
patient 6
CMV copies/ml
CMV specific cells/µl
Median SFU above unstimulated control
% CMV specific of total CD8+ T cells
days post transplantation

Continued

Continued

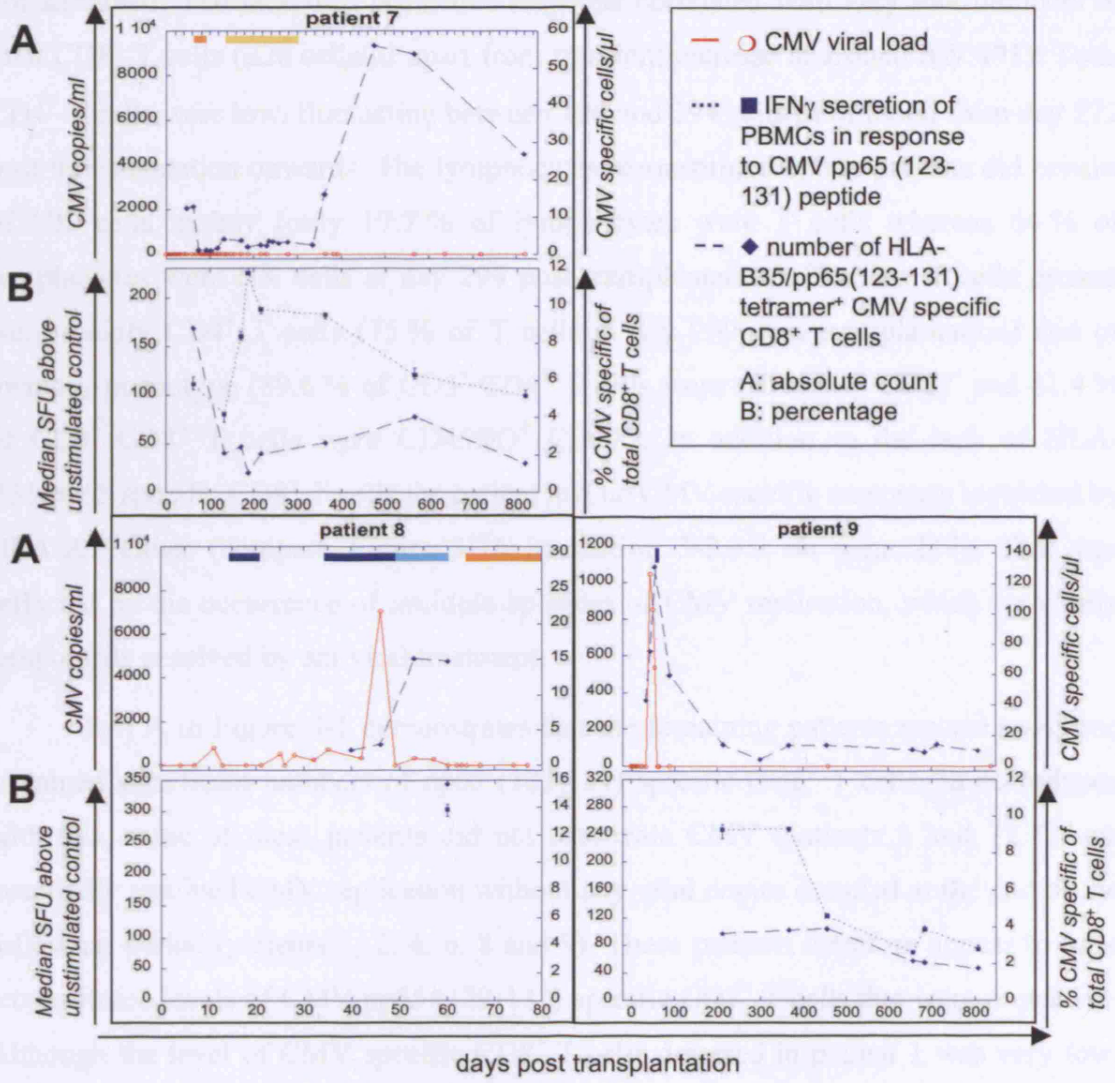


Figure 3-1 Monitoring of HLA-B35/pp65 specific $CD8^+$ T cell responses in patients over time

Responses of different patients are shown in separate boxes. Viral load measurements are illustrated along with the absolute count of HLA-B35/pp65 specific $CD8^+$ T cells in A. Part B illustrates the IFNγ response to CMV pp65 (123-131) peptide (triplicate measurements with the error representing the range from the 25th to the 75th percentiles) along with the HLA-B35/pp65 specific $CD8^+$ T cell response as a percentage of total $CD8^+$ T cells (responses are only shown for time points when ELISpot analysis had been performed). The scale of the y-axis is larger for patients 7 and 9 in A and for patient 8 in B to adjust for higher responses. Horizontal bars in yellow/red or blue indicate immunosuppressive (MMF: mycophenolate mofetil, Prednisolone, Dexamethasone) and antiviral (Ganciclovir, Foscarnet) treatment respectively.

The time course of a patient who failed to demonstrate a protective CMV specific response and who therefore remained at high risk of CMV reactivation is shown by patient 3 in Figure 3-1. The patient had received intense conditioning with Campath-1H *in vivo* and received his transplant from a CMV seronegative donor. A temporarily low

level of CMV specific $CD8^+$ T cells in this patient at 491 days post transplantation was not sustained. The lack of a protective response correlated with very low numbers of total $CD8^+$ T cells (≤28 cells/µl apart from transient increase at around day 491). Total $CD4^+$ T cells were low, fluctuating between 126 and 294 cells/µl of blood from day 272 post transplantation onwards. The lymphocytes reconstituted by the patients did consist of NK cells mainly (only 17.7 % of lymphocytes were T cells whereas 64 % of lymphocytes were NK cells at day 299 post transplantation). The few T cells present were mainly $CD4^+$ T cells (75 % of T cells at day 299 post transplantation) and of memory phenotype (89.6 % of $CD3^+$ $CD4^+$ T cells were $CD45RO^+$ $CD27^-$ and 81.4 % of $CD3^+$ $CD8^+$ T cells were $CD45RO^+$ $CD27^-$). In addition to the lack of HLA-B35/pp65 specific $CD8^+$ T cells the patient had no CMV specific responses restricted by HLA-A1 either (compare Figure 3-16 in section 3-2.3.2 on page 217). This was reflected by the occurrence of multiple episodes of CMV replication, which were only temporarily resolved by antiviral treatment.

Part A in Figure 3-1 demonstrates that the remaining patients reconstituted and sustained significant numbers of pp65 (123-131) specific $CD8^+$ T cells. In accordance with this, some of these patients did not reactivate CMV (patients 5 and 7). Others eventually resolved CMV replication without any viral copies detected at the end of the follow-up period (patients 1, 2, 4, 6, 8 and 9). These patients therefore appear to have reconstituted levels of CMV pp65 (123-131) specific $CD8^+$ T cells that were protective. Although the level of CMV specific $CD8^+$ T cells detected in patient 1 was very low, the genuineness of the response was confirmed by the functional assay, which demonstrated IFNγ release by PBMC upon stimulation with CMV pp65 (123-131) peptide, shown in Figure 3-1.

Some patients expressed several of the HLA types for which CMV specific $CD8^+$ T cells were investigated in this chapter (compare Table 2-1, page 99). These patients, whose HLA-B35/pp65 specific $CD8^+$ T cells responses are shown in this section, were also monitored for the presence of CMV specific $CD8^+$ T cells restricted by other HLA alleles. The latter will be shown in later sections. Of these, patient 6 also reconstituted HLA-A*0201 restricted CMV specific $CD8^+$ T cells (Figure 3-21, page 229) whereas such cells were not observed in patient 1 or patient 2 (Figure 3-23, page 231). Patient 1 and 2 expressed HLA-A*0201 but not HLA-B*0702. Therefore the lack of HLA-A*0201 restricted CMV specific $CD8^+$ T cells cannot be explained by the dominance of CMV specific $CD8^+$ T cell responses restricted by HLA-B*0702 over

those restricted by HLA-A*0201 that was reported previously (Lacey *et al.*, 2003). Furthermore patient 5 reconstituted HLA-A*0101 restricted CMV specific $CD8^+$ T cells (Figure 3-14, page 214) but patient 7 did not reconstitute CMV specific $CD8^+$ T cells restricted by HLA-A*2402 (Figure 3-9, page 201). Possible dominance of CMV specific $CD8^+$ T cells restricted by one HLA allele over those restricted by another HLA allele will be discussed in section 3-3 from page 238.

The protective capacity of HLA-B35/pp65 specific $CD8^+$ T cells was best demonstrated by observations in patient 9. This patient reactivated CMV at 42 days post autologous transplantation. CMV specific T cells were present and their level was rising rapidly until day 53. In accordance with this the viral load had already dropped at day 49 before Ganciclovir was given from day 54 to 62 post transplantation. CMV replication was subsequently not detectable from day 59 post transplantation onwards. HLA-B35/pp65 specific $CD8^+$ T cells therefore most likely caused the initial drop in viral load and protected the patient from subsequent reactivation.

Tetramer staining was used as a direct approach to visualise and quantify CMV peptide pp65 (123-131) specific $CD8^+$ T cells in absolute terms and as a percentage of total $CD8^+$ T cell response in patients. Additionally the functionality of these responses was confirmed by ELISpot assays measuring the IFNγ release after stimulation with pp65 (123-131) as spot-forming units (SFU) per 100,000 PBMC. Some variations between the two assays are expected. If $CD8^+$ T cells represent a different proportion of PBMC in different patients, these patients will have different numbers of SFU observed in the ELISpot assay even if they demonstrate a similar number of CMV specific $CD8^+$ T cells observed by tetramer staining. Nevertheless Figure 3-1 on page 180 demonstrates cytokine release of PBMC from patient blood samples at the time points when pp65 (123-131) specific $CD8^+$ T cell staining of PBMC was positive in these patients. This confirms functionality of responses quantified by tetramer staining.

3-2.1.3 HLA-B35 restricted $CD8^+$ T cell response with potential crossreactivity in patient 7

Of all the patients in whom HLA-B35/pp65 specific $CD8^+$ T cell responses were measured, one was of particular interest. Patient 7 was CMV seropositive. In contrast to other patients of this HLA type specific follow-up cohort this patient received a myeloablative, non-TCD transplant from a CMV seronegative donor (Table 2-1, page 99) and was therefore at greater risk of CMV reactivation (Cwynarski *et al.*, 2001,

Gandhi *et al.*, 2003a) because the contribution of newly generated CMV specific T cells from the naïve T cell pool of donor origin during the first 6 months after transplantation may be negligible (Roux *et al.*, 2000) and recipient T cells typically survive only when the graft is thoroughly depleted of donor T cells (Roux *et al.*, 1992). Nevertheless a high frequency of total $CD8^+$ T cells bound HLA-B35/pp65 tetramer since the first time of measurement at day 46 post transplantation in patient 7. At that time 11 percent of total $CD8^+$ T cells were HLA-B35/pp65 specific. CMV specific T cells of donor origin have recently been demonstrated in a HSCT patient early after receiving a non T cell depleted graft from a CMV seronegative donor and reactivation of CMV (Chalandon *et al.*, 2006). Reactivation of CMV, however, was not evident at any time post transplantation in patient 7. The development of a primary CMV specific response of high frequency was surprising. Two observations in particular indicated that this CMV response might be unusual. First, the pattern of CMV response closely resembled the rising of total T cell numbers during a period of acute GvHD and second the HLA-B35/pp65 specific $CD8^+$ T cells demonstrated an unusual staining profile.

Soon after engraftment at day 17 post transplantation, acute GvHD of grade 1 - 2 was diagnosed from day 21 post transplantation onwards. The patient subsequently received increased levels of Cyclosporine from day 42 post transplantation and additional Prednisolone treatment from day 62 to 94 post transplantation. CMV specific and total T cell numbers were initially high and dropped in parallel with the immunosuppressive treatment given after day 62 post transplantation. Cell numbers recovered after day 117 post transplantation. Prophylactic Cyclosporine treatment had been reduced from day 222 and stopped at day 249. Mycophenolate Mofetil treatment was given for chronic GvHD from day 136 to 301. Subsequently CMV specific $CD8^+$ T cells increased after day 335 post transplantation. An unusually dim tetramer staining profile of HLA-B35/pp65 specific $CD8^+$ T cells was observed during the first period of follow-up until day 363 post transplantation and is shown in Figure 3-2.

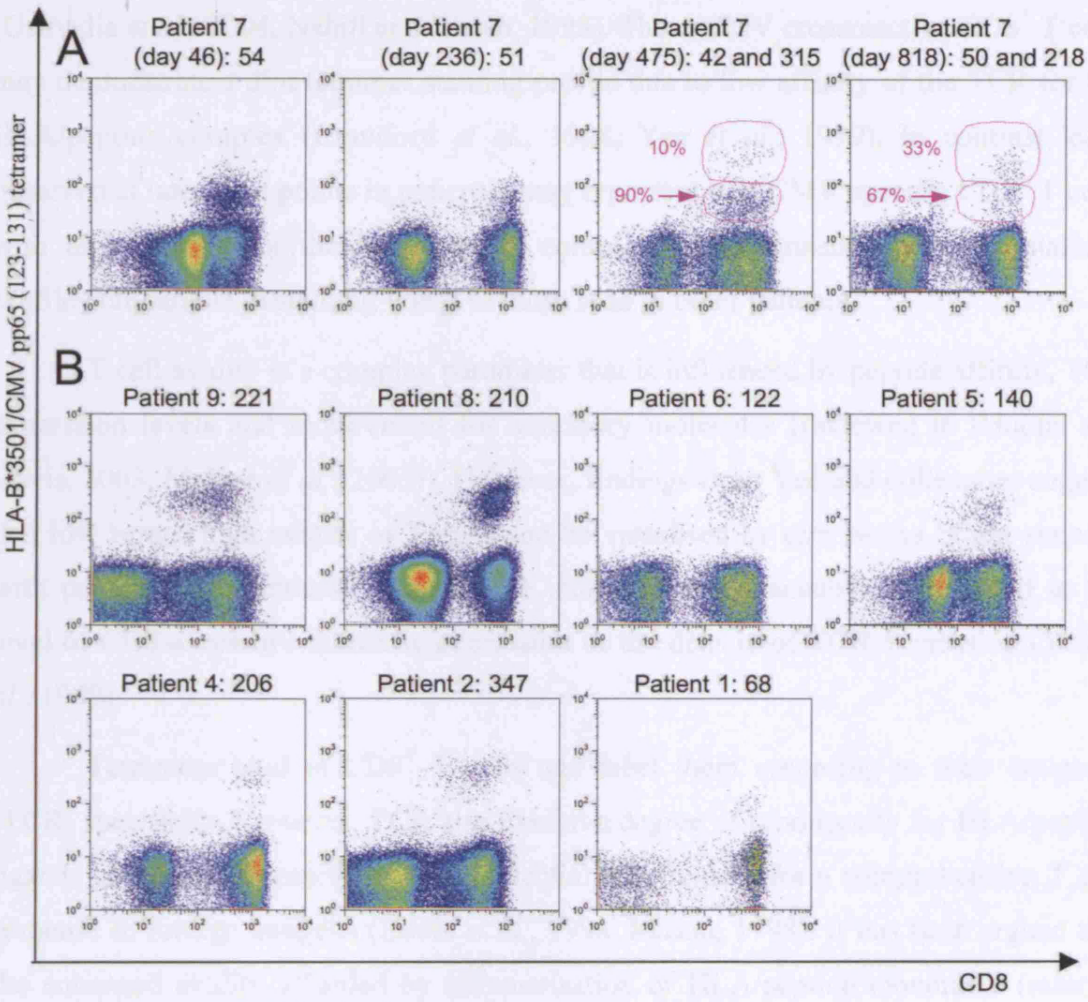


Figure 3-2 Dim and bright tetramer staining of HLA-B35/pp65 specific CD8$^+$ T cells in patient 7

Representative examples of tetramer stained PBMC from patient 7 are shown in A in comparison to typical staining profiles from all other patients of this follow-up cohort who demonstrated significant numbers of tetramer binding cells in B. Flow cytometry plots show CD3$^+$ live lymphocytes sorted according to their surface expression of CD8 on the x-axis and HLA-B35/pp65 tetramer staining on the y-axis. The geometrical mean of HLA-B35/pp65 tetramer staining intensity is given above each plot. For samples from patient 7 at day 475 and day 818 post transplantation, the geometrical mean of dim and bright staining populations as marked by pink circles is shown.

After day 363 post transplantation CMV specific CD8$^+$ T cells were present in higher numbers per volume of blood than before (Figure 3-1, page 180) and demonstrated two populations of an unusually dim and a normal bright tetramer staining profile respectively (Figure 3-2). The latter staining profile resembled the staining levels of CMV specific CD8$^+$ T cells observed in other patients. These observations in patient 7 led to the hypothesis that alloreactive T cells present at the time of GvHD symptoms in the patient, may have cross-reacted with the HLA-B35/pp65 tetramer. The possibility of CMV specific CD8$^+$ T cells cross-reacting with alloantigen was reported previously

(Gamadia *et al.*, 2004, Nahill and Welsh, 1993). These CMV crossreactive $CD8^+$ T cells may demonstrate a dim tetramer staining profile due to low affinity of the TCR for the HLA/peptide complex (Crawford *et al.*, 1998, Yee *et al.*, 1999). In contrast, cells observed at later time points in patient 7 may represent true CMV specific $CD8^+$ T cells with high affinity for the HLA/peptide complex that demonstrate a bright staining profile comparable in staining levels to those seen in other patients.

T cell avidity is a complex parameter that is influenced by peptide affinity, TCR expression levels and requirement for accessory molecules (reviewed in (Huppa and Davis, 2003, McKee *et al.*, 2005)). However, findings from Yee and colleagues suggest that low *versus* high avidity of T cells can be visualised by dim *versus* bright staining with peptide-MHC tetramer and that the underlying mechanism is not related to the level of CD8 accessory molecule expression or the density of TCR expression (Yee *et al.*, 1999).

Tetramers bind to $CD8^+$ T cells and label them according to their antigenic (TCR) specificity. However, TCR also exhibit a degree of promiscuity for HLA/peptide ligands and this crossreactivity is an essential requirement for a comprehensive T cell response to foreign antigens (Davis *et al.*, 1998, Mason, 1998). It has been argued that the enhanced avidity afforded by tetramerisation of HLA/peptide monomers (refer to section 1-10) may allow tetramer binding to TCR with low affinities that would not generate physiological responses (Howard *et al.*, 1999). The crossreactivity of tetramers was correlated to the temperature used during tetramer staining procedures (Whelan *et al.*, 1999). Strongly recognised (high affinity) HLA/peptide complexes are thought to interact long enough to trigger the TCR but rapidly enough to be available for several cycles of binding (Lanzavecchia *et al.*, 1999) that may be needed for full T cell activation (Valitutti *et al.*, 1995). Just as the ability of HLA/peptide complexes to activate T cells depends on their off-rate (Lanzavecchia *et al.*, 1999), the ability of tetramers to stain T cells depends upon a sufficiently slow off-rate (Whelan *et al.*, 1999). Staining at high temperature likely increases the off-rate beyond the threshold at which tetramers incorporating weakly recognised HLA/peptide can form stable complexes with a TCR. Staining at low temperature may allow for sufficient interaction of tetramers incorporating weakly recognised HLA/peptide to result in stable binding to a TCR despite an inability of these HLA/peptide complexes to activate T cells (Whelan *et al.*, 1999). Therefore tetramer staining during this study was performed at a temperature of 37 °C that was reported to result in reduced staining by tetramers

incorporating weakly recognised HLA/peptide without reducing the staining by tetramers incorporating strongly recognised HLA/peptide (refer to section 2-5.1). Staining at that temperature shows a very good correlation with peptide activity in cytotoxicity assays, which suggests that it can accurately reflect the specificity of T cells for HLA/peptide presented on the cell surface at physiological levels (Burrows *et al.*, 2000). This, however, cannot preclude crossreactivity similar to that, which would generate physiological crossreactive responses. Therefore $CD8^+$ T cells may bind tetramer because they possess TCR with concise specificity to the HLA/peptide that is incorporated in the tetramer or because they possess TCR with specificity to a foreign HLA/peptide complex but sufficient avidity to crossreact with the tetramer.

T cell avidity can be tested *in vitro* by evaluating the peptide amounts required for induction of a T cell response (reviewed in (Snyder *et al.*, 2003)). Cytokine secretion in response to CMV pp65 (123-131) by PBMC from patient 7 had been observed during the entire period of follow-up (Figure 3-1, page 180). Therefore affinity of PBMC was tested by ELISpot assay using titration of pp65 (123-131). PBMC from patient 7 early post transplantation (day 77, when CMV specific T cells demonstrated a dim tetramer staining profile) and samples from the same patient late post transplantation (day 818, when CMV specific T cells demonstrated a dim and bright tetramer staining profile) were compared to samples from other patients with evident HLA-B35/pp65 specific $CD8^+$ T cells. Response curves are shown as percentage of responses to undiluted peptide in Figure 3-3.

A variation of this graph (Figure 6-3, page 335) illustrating original median values and errors calculated from triplicate measurements is shown in the appendix.

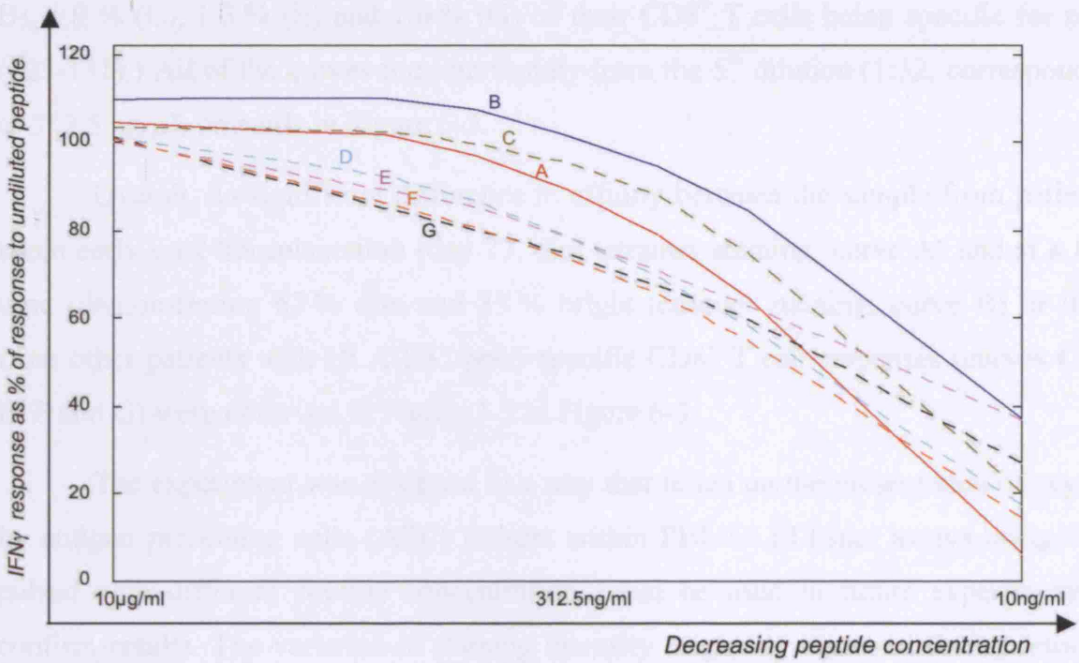


Figure 3-3 IFNγ release of PBMC in response to serial dilutions of CMV peptide pp65 (123-131)

IFNγ release in responses to 1:2 dilutions of CMV pp65 (123-131) was measured in triplicate for two PBMC samples from patient 7 (solid lines, A: sample taken at day 77, B: sample taken at day 818 post transplantation) and five samples from other patients (dashed lines, C: patient 30b at 763 days post transplantation, D: patient 31 at 700 days post transplantation, E: patient 6 at 86 days post transplantation, F: patient 9 at 367 days post transplantation and G: patient 32 at 483 days post transplantation). A weighted curve fit (75 % smoothing in the program Kaleidagraph) from the median SFU above unstimulated controls from triplicate measurements was calculated for each patient sample. For clarity of the graphical display individual data points are not shown but can be seen in a variation of this graph (Figure 6-3) in the appendix. Response curves are represented as percent of response to the highest concentration of peptide (10 µg/ml).

Not all response curves in this figure demonstrate 100 % of response towards undiluted peptide. Cells from some of the samples demonstrated higher responses to middle range dilutions of peptide than to undiluted peptide. This may be the result of general fluctuation of data points around the titration trend line reflecting the limits of the ELISpot sensitivity. Alternatively it may be the result of a prozone effect that may have been caused for example by a mild toxic effect of high peptide concentrations.

Figure 3-3 suggests that cells from patients 7 and 31 titre out slightly faster than other responses (Figure 3-3: A, B, and C). This, however, cannot be observed when original data points (instead of percentages of the neat response) are plotted (Figure 6-3 in the appendix, page 335). That figure shows higher initial responses in curve D and F, which derive from samples with 2.6 % and 3.8 % of their $CD8^+$ T cells being specific for pp65(123-131), than in other curves, which derive from samples with 1.5 % (A and

B), 1.9 % (C), 1.3 % (E) and 1.0 % (G) of their $CD8^+$ T cells being specific for pp65 (123-131).) All of the curves titer out rapidly from the 5th dilution (1:32, corresponding to 312.5 ng/µl) onwards in Figure 6-3.

Overall, no significant difference in affinity between the sample from patient 7 taken early post transplantation (day 77, dim tetramer staining, curve A) and at a later time (demonstrating 67 % dim and 33 % bright tetramer staining, curve B) or those from other patients with HLA-B35/pp65 specific $CD8^+$ T cell responses (curves C, D, E, F and G) were observed in Figure 3-3 or Figure 6-3.

The experiment was designed in a way that relied on the presentation of peptide by antigen presenting cells (APC) present within PBMC. ELISpot assays using APC pulsed with different peptide concentration could be used in future experiments to confirm results. The variation in staining intensity originally observed during tetramer staining may reflect a rapid dissociation rate for TCR-peptide/MHC interaction. This may result in a lower proportion of tetramer bound TCRs from low affinity T cells at a given time reflected in a lower fluorescent intensity than from high affinity T cells. This could be investigated by receptor-ligand binding assays in future studies.

A further observation suggests that binding of tetramer stained cells may be different for the cells that appear as a dim tetramer stained population in comparison with those that appear as a bright tetramer stained population in Figure 3-2 on page 184. As described in section 2-5.3, binding specificity of tetramers was confirmed by decreased CD3 fluorescence intensity in the tetramer staining populations in comparison to the total $CD8^+$ T cell populations during flow cytometry (Figure 2-5, page 111). Interestingly, cells from patient 7 at day 818, demonstrated less CD3 fluorescence in the bright tetramer populations (107 fluorescence units) than in the dim tetramer populations (112 fluorescence units). Both populations had less CD3 fluorescence than the total $CD8^+$ T cell population (114 fluorescence units).

Low cell numbers were available from this patient. Further experiments such as using an *in vitro* skin explant model (Sviland and Dickinson, 1999) would have been possibilities to further explore whether the dim staining cells observed in patient 7 may be true CMV specific $CD8^+$ T cells or crossreactive. Due to the limited material available, however, the staining results from this patient can serve as an observation only. The hypothesis on dim tetramer binding cells being allo-crossreactive could not be proven and the significance of the unusual staining profiles observed in patient 7 remains unknown. Responses from this patient are therefore considered similar to

responses of other patients within the follow-up cohort for subsequent statistical analysis to establish protective response levels in the context of HLA-B35.

3-2.1.4 Statistical analysis of the protective level of HLA-B35 restricted CMV specific CD8$^+$ T cells and summary

The numbers of CMV specific CD8$^+$ T cells measured per volume of peripheral blood (compare Figure 3-1 on page 180, upper graph in each box) were analysed statistically. CMV specific CD8$^+$ T cell numbers measured in all patients were sorted into three groups. One group contained responses from patients that did not experience CMV reactivations. Responses from other patients were separated according to the occurrence of CMV reactivations during the time of measurement. Responses measured from the last peak of CMV reactivation onwards were placed into a second group, whereas those measured prior to the last CMV reactivation peak were considered in a third group. The assignment of responses to these three groups can be explained as follows. A lack of responses in patients not experiencing CMV reactivations may occur but in general the level of CMV specific CD8$^+$ T cells in patients who never reactivate is considered protective in this analysis. The level of CMV specific CD8$^+$ T cells in patients during periods of CMV reactivation is likely to be not protective although these responses may include peak levels of protective responses that are subsequently not sustained. In contrast, the level of responses measured since the last CMV reactivation may be considered to be protective. The three groups of responses were statistically analysed using a two-tailed Mann-Whitney U test. The results are shown in Figure 3-4 with the medians indicated as lines.

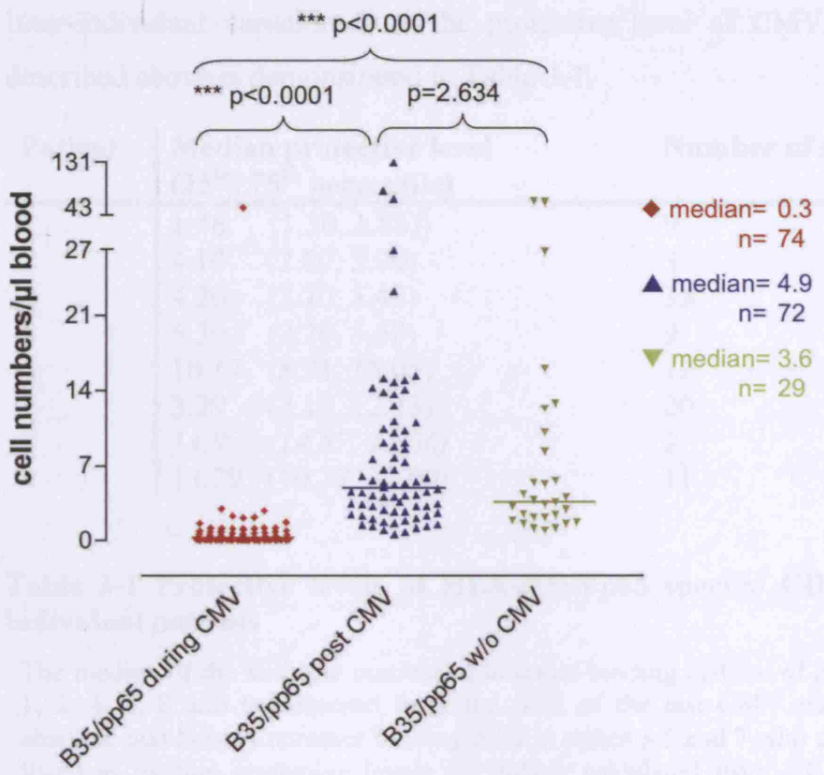


Figure 3-4 Statistical analysis of HLA-B35/pp65 specific CD8$^+$ T cell responses

The figure shows absolute numbers of tetramer binding cells/µl of peripheral blood. Compared are values in patients 1, 2, 3, 4, 6, 8 and 9 measured post transplantation until the last observed CMV reactivation (◆B35/pp65 during CMV) *versus* values measured from the peak of the last CMV reactivation onwards in these patients (▲B35/pp65 post CMV) and values in patients 5 and 7 who did not reactivate CMV (▼B35/pp65 w/o CMV). Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

The nine patients of the follow-up cohort can be grouped into the 7 patients who reactivated CMV during the monitoring period (patients 1, 2, 3, 4, 6, 8 and 9) and the two patients who did not (patients 5 and 7). The median of CMV specific CD8$^+$ T cell numbers measured in patients who did not reactivate the virus was 3.6 cells/µl. CMV specific CD8$^+$ T cell numbers measured during the period of CMV reactivation (median 0.3 cells/µl) were significantly lower than cell numbers measured after CMV reactivation in the reactivating group of patients (median 4.9 cells/µl) and cell numbers measured in patients who did not reactivate the virus. The latter two response groups were not significantly different from each other. This demonstrates a broad inverse correlation between the HLA-B35/pp65 specific CD8$^+$ T cell numbers and the viral load in HSCT patients. Significantly higher CMV specific CD8$^+$ T cell numbers were detected in patients that had resolved CMV reactivations than during the period of CMV reactivation. The magnitude of responses in patients after resolution of CMV was similar to the magnitude of responses in patients that did not reactivate CMV.

Inter-individual variation from the protective level of CMV specific $CD8^+$ T cells described above is demonstrated in Table 3-1.

Patient	Median protective level (25^{th}, 75^{th} percentile)	Number of samples
1	1.46 (1.30, 1.86))	9
2	4.10 (2.80, 5.00)	3
4	4.20 (2.70, 5.45)	35
5	5.30 (2.20, 5.60)	9
6	10.17 (8.21, 15.05)	12
7	3.29 (2.13, 12.43)	20
8	*14.97 (14.87, 15.06)*	*2*
9	13.79 (10.77, 37.00)	11

Table 3-1 Protective levels of HLA-B35/pp65 specific $CD8^+$ T cell responses in individual patients

The median of the absolute number of tetramer binding cells/µl of peripheral blood in patients 1, 2, 4, 6, 8 and 9 measured from the peak of the last CMV reactivation onwards and of absolute numbers of tetramer binding cells in patients 5 and 7 who did not reactivate CMV are listed as median protective levels (in italics: calculated from <3 samples). The number of samples used to calculate the median is shown on the right.

Patient 3 is not included in Table 3-1 because multiple CMV reactivations spanning the entire time of follow-up indicated that protective levels of HLA-B35/pp65 specific $CD8^+$ T cell numbers were not reached in this patient. Apart from a temporary peak of 2.14 cells/µl, this patient demonstrated ≤0.14 CMV specific $CD8^+$ T cells/µl blood (Figure 3-1, page 180).

Table 3-1 demonstrates higher responses in patients 6, 8 and 9 than in other patients. In patient 6 this results from peak levels. He recovered 4.1 HLA-B35/pp65 specific $CD8^+$ T cells/µl blood post CMV reactivation with intermittent spikes in his response. In patient 8 HLA-B35/pp65 specific $CD8^+$ T cells were measured from 2 samples post CMV reactivation only before the patient died. This response most likely represented the peak levels of $CD8^+$ T cells, which numbers may have decreased thereafter. High levels of HLA-B35/pp65 specific $CD8^+$ T cells were also observed in patient 9. After an initial spike of the response, subsequent levels decreased to 4.44 cells/µl blood, which did plateau at around 12 cells/µl blood thereafter. Patient 9 was the only autologous patient in this cohort (compare Table 2-1 on page 99). This may explain the high level of CMV specific $CD8^+$ T cell numbers observed in this patient because it was reported previously that CMV specific $CD8^+$ T cells expand to much greater number in autologous than allogeneic HSCT patients early post

transplantation. This was suggested to be due to activation of allo-transferred cells, which are more prone to apoptosis than auto-transferred cells (Ferrari *et al.*, 2006).

Calculating the median from the levels shown in Table 3-1 results in a value of 4.75 cells/µl of blood, which lies between the level of responses measured in all patients since the last peak of CMV reactivation (median 4.9, Figure 3-4. page 190) and the level of responses measured in all patients who did not reactivate CMV (median 3.6, Figure 3-4). It thus confirms that the calculation of the protective level of responses is similar for both approaches, the calculation using measurements from all patient samples (with varying samples numbers from different patient) and the calculation using a single calculated value per patient.

It can be concluded that HLA-B35/pp65 specific $CD8^+$ T cells are protective at a level of approximately $4.4x10^6$ cells/L of blood (Figure 3-26, page 241). This final value has been established by calculating the median of responses measured in patients 1, 2, 4, 6, 8 and 9 since the last peak of CMV reactivation and all responses measured in patients 5 and 7. This value would only slightly change ($5x10^6$ cells/L of blood) if responses from patient 7 were omitted (compare section 3-2.1.3 from page 182 onwards). Section 3-3 from page 238 onwards will show that the protective level of HLA-B35/pp65 specific $CD8^+$ T cells established here is considerably lower than for CMV specific $CD8^+$ T cells targeting other HLA/peptide combinations such as HLA-A*0201 restricted CMV pp65 (495-503) specific responses.

Low levels of HLA-B35/pp65 specific $CD8^+$ T cell responses can also be observed in healthy CMV seropositive volunteers. Six CMV seropositive healthy individuals expressing HLA-B*3501 (compare Table 2-3 on page 100) were recruited and their blood tested for HLA-B35/pp65 specific $CD8^+$ T cells. The frequency of CMV specific cells amongst $CD8^+$ T cells was found to be 0.27 % in one individual and ≤0.07 % in another two of the six individuals tested. Discrete populations can be observed in flow cytometry plots in the two patients demonstrating very low percentages of CMV specific $CD8^+$ T cells (Figure 3-5). The remaining three individuals had no detectable HLA-B35/pp65 specific $CD8^+$ T cells.

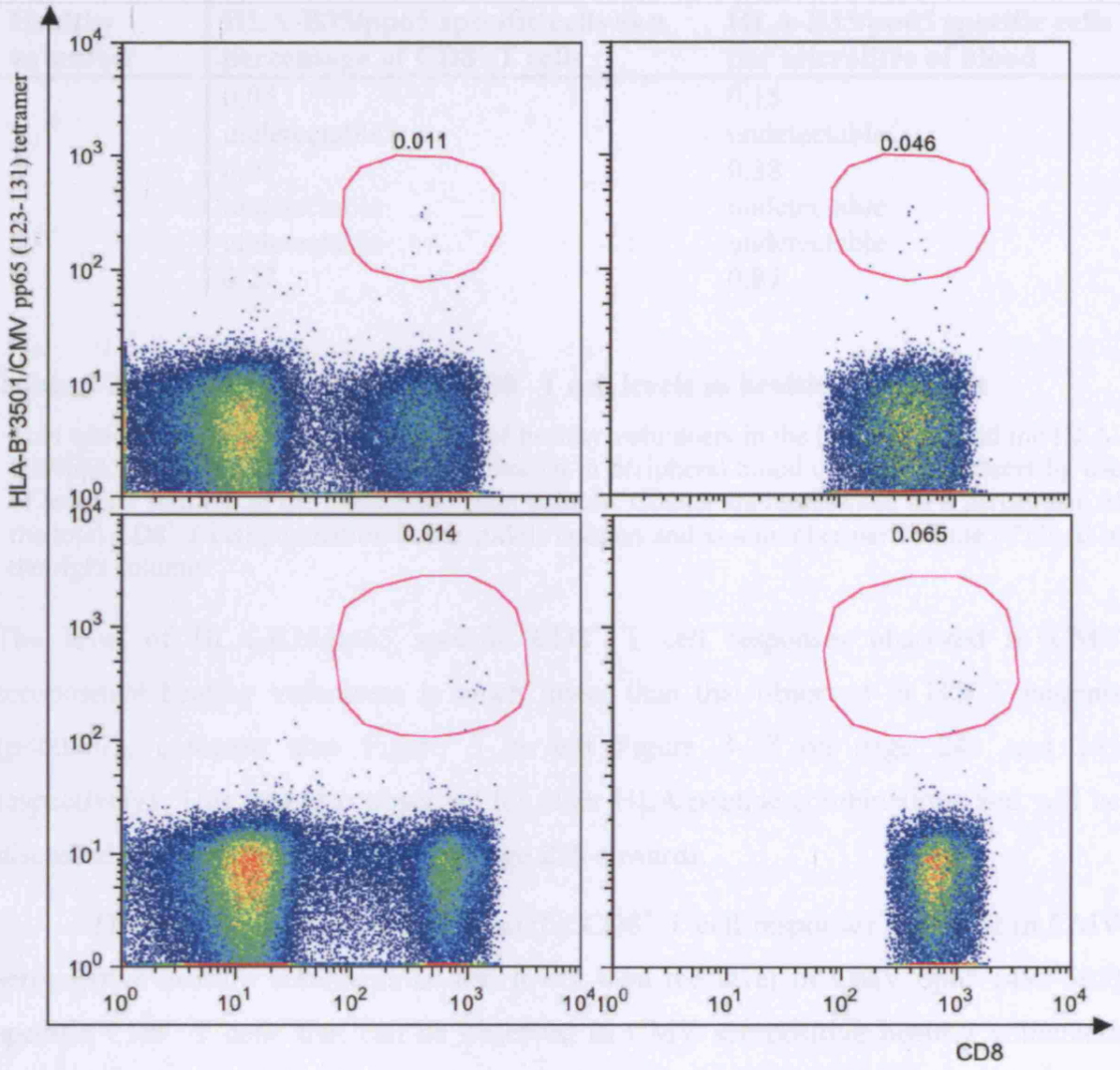


Figure 3-5 Staining profiles of HLA-B35/pp65 specific CD8$^+$ T cells of low frequency in healthy volunteers 1 and 3

The figure shows tetramer stained peripheral blood cells from two healthy volunteers. Flow cytometry plots show cells sorted depending on their surface expression of CD8 and HLA-B35/pp65 tetramer staining. Plots on the left represent CD3$^+$ live lymphocytes. Plots on the right represent the CD8$^+$ fraction of these cells. Numbers in the upper right corner of plots represent the cells shown in pink circular gates as a percentage of total cells in that plot.

A summary of all levels of HLA-B35/pp65 specific CD8$^+$ T cells observed in healthy volunteers is demonstrated in Table 3-2.

Healthy volunteer	HLA-B35/pp65 specific cells as a percentage of $CD8^+$ T cells	HLA-B35/pp65 specific cells per microlitre of blood
1	0.05	0.15
2	undetectable	undetectable
3	0.07	0.38
4	undetectable	undetectable
5	undetectable	undetectable
6	0.27	0.87

Table 3-2 HLA-B35/pp65 specific $CD8^+$ T cell levels in healthy volunteers

This table shows identification numbers of healthy volunteers in the left column and the HLA-B35/pp65 specific $CD8^+$ T cell counts detected in peripheral blood of these volunteers by use of tetramer staining in the middle and right column. Counts are represented as a percentage of the total $CD8^+$ T cell population in the middle column and as a number per volume of blood in the right column.

The level of HLA-B35/pp65 specific $CD8^+$ T cell responses observed in CMV seropositive healthy volunteers is much lower than that observed in HSCT patients ($p<0.0001$, compare also Figure 3-26 and Figure 3-27 on page 241 and 245 respectively). This was also observed for other HLA/peptide combinations and will be discussed further in section 3-3 from page 238 onwards.

The level of HLA-B35/pp65 specific $CD8^+$ T cell responses observed in CMV seropositive healthy volunteers is also lower than the level of CMV pp65 (495-503) specific $CD8^+$ T cells that can be observed in CMV seropositive healthy volunteers expressing HLA-A*0201 (compare Table 3-11, page 237).

A range of other CMV specific $CD8^+$ T cell responses targeting different HLA/peptide combinations will be compared to HLA-A*0201 restricted CMV pp65 (495-503) specific responses in the following sections.

3-2.2 CMV specific $CD8^+$ T cell responses restricted by HLA-A24

In many ethnic groups, HLA-A24 is one of the most common HLA types with HLA-A*2402 being the most frequent allele (compare section 1-11.1). HLA-A*2402 restricted CMV specific $CD8^+$ T cells have been studied mainly by Japanese groups (Akiyama *et al.*, 2002, Kuzushima *et al.*, 2001, Masuoka *et al.*, 2001, Morita *et al.*, 2005, Sakagawa *et al.*, 2006) because the HLA-A*2402 allele has a much higher gene frequency (32.7 %) than HLA-A*0201 (10.6 %) in Japan (Tokunaga *et al.*, 1997) and is represented in 58 % of Japanese (Sette and Sidney, 1999).

This section will demonstrate that low levels of CMV specific $CD8^+$ T cells restricted by HLA-A*2402 protect HSCT patients from CMV reactivation. Two CMV

peptide targets studied in the context of this allele will be described first. This will be followed by results indicating that one of these targets does not induce $CD8^+$ T cell responses of significant frequency in HSCT patients. Longitudinal monitoring of $CD8^+$ T cells specific for the second target will be shown for individual patients. Subsequently assignment of responses to different groups according to viral load in patients will be performed. The protective level of $CD8^+$ T cells to the second target will then be established by statistical comparison of these groups. Finally levels in patients and healthy volunteers will be summarised and a comparison to HLA-A*0201 restricted CMV pp65 (495-503) specific responses will be shown in section 3-3 from page 238 onwards.

3-2.2.1 CMV targets of $CD8^+$ T cell responses restricted by HLA-A24

This section describes HLA-A*2402 restricted $CD8^+$ T cell responses directed towards CMV pp65. CMV pp65 peptides targeted by $CD8^+$ T cells restricted by HLA-A*2402 had been identified immediately prior to the start of this project. The peptide FTSQYRIQGKL at position pp65 369-379 is an 11mer originally described by Longmate and colleagues (Longmate *et al.*, 2001). A study by Kuzushima and colleagues aiming at identifying pp65 peptides restricted by HLA-A*2402, however, did not confirm responses to this peptide (Kuzushima *et al.*, 2001). They used computer-assisted algorithms, MHC stabilization and enzyme linked immunospot assays to identify peptides and demonstrated a highly focused response towards the peptide QYDPVAALF located at the pp65 341-349 region. Endogenous processing and presentation of pp65 (341-349) was confirmed using cytotoxic assays in addition to flow cytometry based techniques. Interestingly, this peptide was shown to bind to HLA-Cw*0401 as well as HLA-A*2402 (Kondo *et al.*, 2003). Yet another study suggested that $CD8^+$ T cell responses to this peptide do occur also in the context of HLA-A*0101 (Ghei *et al.*, 2005), which will be discussed in the next section (3-2.3 from page 206). The peptide VYALPLKML, which is located at the pp65 113-121 region, was also recognised by CMV specific $CD8^+$ T cell lines in the study by Kuzushima and colleagues but only at concentrations higher than pp65 (341-349). The peptide pp65 (113-121) was also found to bind HLA-A24 and induce $CD8^+$ T cell responses independently by another group of scientists (Masuoka *et al.*, 2001). It was therefore decided to investigate $CD8^+$ T cell responses to both, pp65 (341-349) and pp65 (113-121), in this study.

3-2.2.2 Monitoring of CMV specific CD8$^+$ T ceils in patients expressing HLA-A24

16 HLA-A*2402 positive HSCT patients, who each received transplants from HLA-A*2402 positive donors were recruited for the study of HLA-A*2402 restricted CMV pp65 (341-349) and pp65 (113-121) specific CD8$^+$ T cell responses. Patient, donor, or both were CMV seropositive. Detailed patient characteristics are given in Table 2-1 on page 99. Patients from whom at least 4 samples were collected starting at less than 100 days post transplantation were included in the follow-up cohort (n = 12) in order to establish the threshold for a protective level of responses to this HLA/peptide target. The average start of follow- up was day 45 (range day 11 - 99) and the average end of follow-up was day 370 (range day 70 - 830) post transplantation with an average of 13 samples taken per patient (range 5 - 35). Numbers of tetramer binding CD8$^+$ T cells were correlated with viral load and functionality of cells was tested by IFNγ assays for some of the samples with sufficient cell numbers.

CD8$^+$ T cell responses targeted to CMV pp65 (113-121) were absent or very low in most patients. Antigen specific T cell numbers lie below a level of 1.12 cells/μl of peripheral blood in all patients of the HLA-A24 follow-up cohort. This is demonstrated in Figure 3-6.

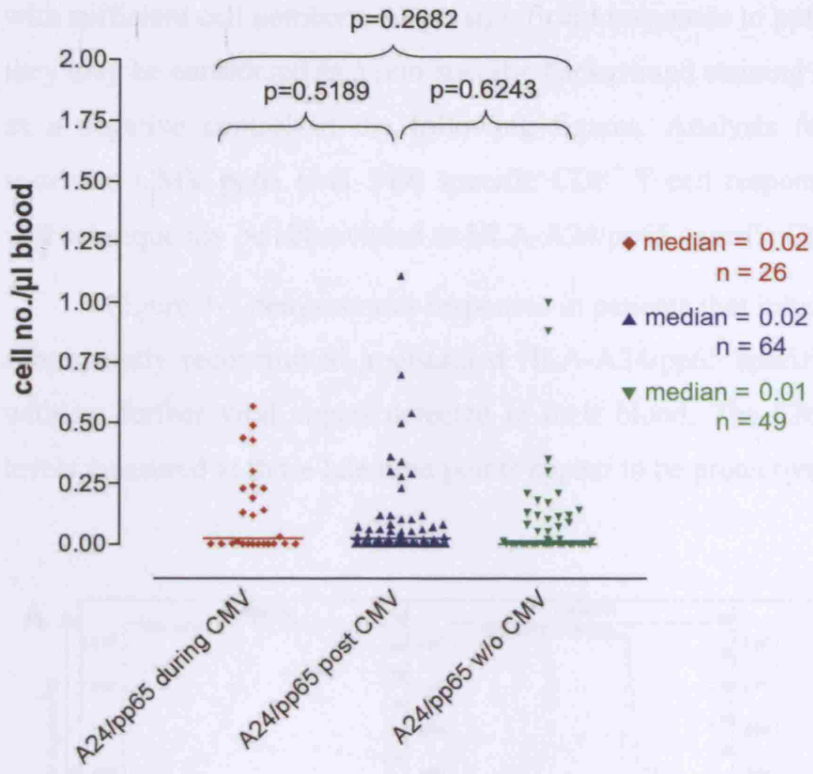


Figure 3-6 Background levels of HLA-A*2402 restricted CMV pp65 (113-121) specific CD8$^+$ T cell responses

The figure shows absolute numbers of HLA-A*2402/pp65 (113-121) tetramer binding cells/µl of peripheral blood in patients of the HLA-A24 follow-up cohort. The scale of the y-axis is over 60 times lower than that of Figure 3-4 on page 190. Groups represent responses measured post transplantation until the last observed CMV reactivation (◆A24/pp65 during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in patients 10 to 12 and 17 to 19 (▲A24/pp65 post CMV) and absolute numbers of tetramer binding cells in patients 7 and 13 to 16, who did not reactivate CMV (▼A24/pp65 w/o CMV). Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

This graph does not include patient 20, who was CMV seronegative and had no detectable CMV replication after receiving a transplant from a CMV seropositive donor suggesting that primary infection did not occur. According to experiences from comparing these staining results with results obtained from staining of control samples, the HLA-A*2402/CMV pp65 (113-121) tetramer has a lower sensitivity (resulting in higher background staining) than that observed with other tetramers including the HLA-B35/pp65 specific tetramer mentioned in the previous section. Most of the HLA-A*2402/CMV pp65 (113-121) specific CD8$^+$ T cell responses measured are at the limit of staining specificity. No significant absolute numbers can be observed in Figure 3-6.

Responses to CMV pp65 (113-121) and pp65 (341-349) were correlated to viral load in patients and the functionality of cells was tested using an IFNγ assay for samples

with sufficient cell numbers. As no significant responses to pp65 (113-121) were found, they may be considered as a non-specific background staining of tetramer and therefore as a negative control in the following figures. Analysis focuses on HLA-A*2402 restricted CMV pp65 (341-349) specific $CD8^+$ T cell responses from here on, which will subsequently be abbreviated as HLA-A24/pp65 specific $CD8^+$ T cell responses.

Figure 3-7 demonstrates responses in patients that initially reactivated CMV but subsequently reconstituted a sustained HLA-A24/pp65 specific $CD8^+$ T cell response with no further viral copies detected in their blood. The CMV specific $CD8^+$ T cell levels measured at those late time points appear to be protective in patients 10 to 12.

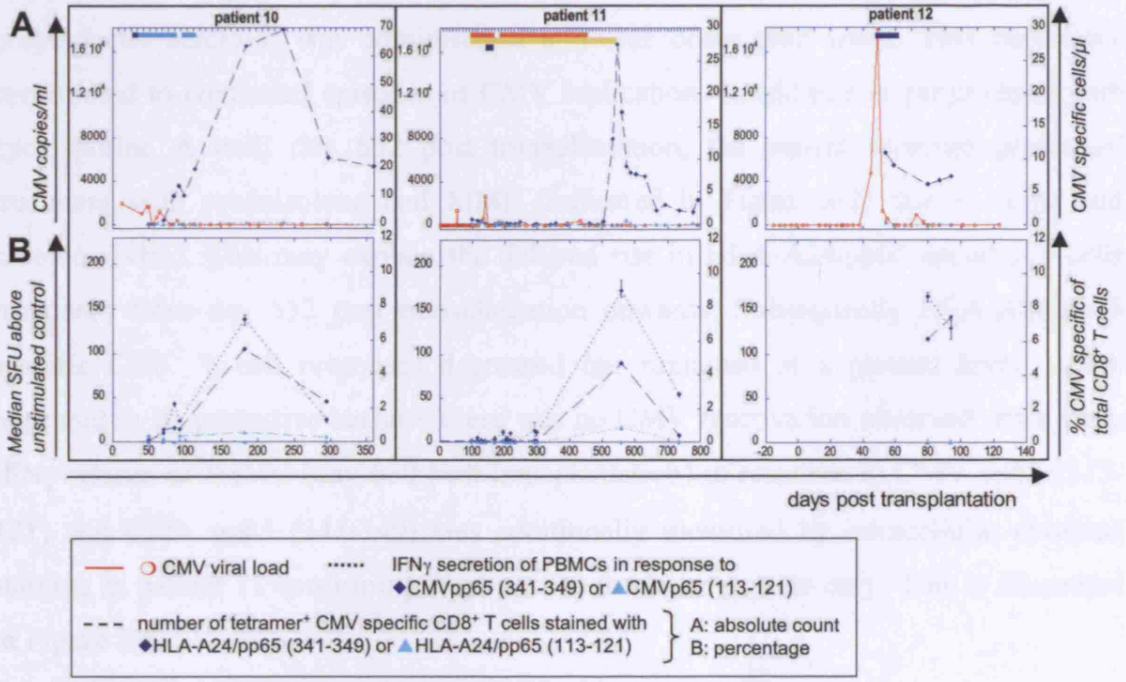


Figure 3-7 Monitoring of HLA-A24/pp65 specific $CD8^+$ T cell responses in patients 10 to 12

◆ HLA-A24/pp65(341-349) and ▲ HLA-A24/pp65(113-121) tetramer binding $CD8^+$ T cells in different patients are shown in separate boxes. Viral load measurements are shown along with the absolute count of CMV specific $CD8^+$ T cell responses in A. Part B indicates the IFNγ response to CMV peptide (triplicate measurements with the error representing the range from the 25th to the 75th percentiles) along with CMV specific $CD8^+$ T cell response as a percentage of the total $CD8^+$ T cell response (responses are only shown for time points when ELISpot analysis had been performed). The scale of the right y-axis is larger for patient 10 in A to adjust for higher responses. Horizontal bars in yellow/red or blue indicate immunosuppressive (MMF: mycophenolate mofetil, Prednisolone) and antiviral (Ganciclovir, Foscarnet) treatment respectively.

Patient 10 had received antithymocyte globulin (ATG) as one of the conditioning regimens prior to transplantation (Table 2-1, page 99). This reagent has a potent capacity to deplete T cells but has been shown to decline to sub-therapeutic levels by 1 month after allogeneic HSCT (Waller *et al.*, 2003). Initial CMV reactivations during the first months post transplantation in patient 10 were treated with antiviral drugs. After cessation of the latter, HLA-A24/pp65 specific $CD8^+$ T cell numbers increased and were sustained at high level until the end of the follow-up period at approximately 1 year post transplantation. No further CMV replication was detected in this patient.

Patient 11 received aciclovir as CMV prophylaxis during nearly the entire time of follow-up. Aciclovir induced encephalopathy, however, necessitated temporary administration of ganciclovir from day 141 to 169 post transplantation and subsequently prophylactic aciclovir was administered at lower doses than usual. This may have contributed to continued episodes of CMV replication. In addition to prophylaxis with cyclosporine A until day 602 post transplantation, the patient received prolonged treatment with prednisolone and MMF (indicated in Figure 3-7) due to acute and chronic GvHD. This may explain the delayed rise in HLA-A24/pp65 specific T cells responses from day 532 post transplantation onwards. Subsequently HLA-A24/pp65 specific $CD8^+$ T cell responses decreased but remained at a plateau level, which appeared to be protective because there was no CMV reactivation observed since then. IFNγ release of PBMC (day 630 post transplantation) in response to CMV pp65 (113-121) and CMV pp65 (341-349) was additionally measured by intracellular cytokine staining in patient 11 confirming responses to the latter peptide only. This is illustrated in Figure 3-8.

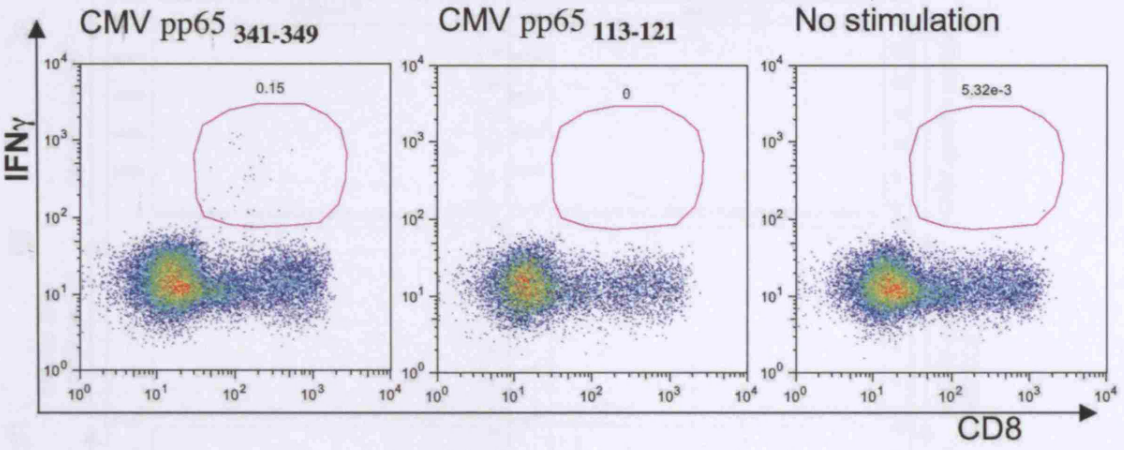


Figure 3-8 Intracellular staining confirming the presence of PBMC responses to pp65 (341-349) but not pp65 (113-121) in patient 11

This figure shows flow cytometry plots illustrating staining for intracellular IFNγ in PBMC (gated on live $CD3^+$ lymphocytes) from patient 11 (630 days post transplantation) that were stimulated with pp65 (341-349), pp65 (113-121) or left unstimulated.

Patient 12 reconstituted high levels of HLA-A24/pp65 specific $CD8^+$ T cell responses after his first CMV reactivation, which was treated with Ganciclovir. The CMV specific T cell level reconstituted most likely cleared the subsequent low-level reactivation, for which no antiviral treatment was received. HLA-A24/pp65 specific $CD8^+$ T cells appear to be protective as no further viral load was detected in the patient thereafter.

Except patients 10 to 12 (Figure 3-7) no other patients of the follow-up cohort (n = 12) reconstituted significant sustained numbers of HLA-A24/pp65 specific $CD8^+$ T cells. 4 of these other 9 patients demonstrated reconstitution of high levels of $CD8^+$ T cell responses specific for other HLA/CMV peptide targets. This is illustrated in Figure 3-9. These patients were protected by responses other than HLA-A24/pp65 specific $CD8^+$ T cells and no CMV replication was observed.

The other 5 (patients 16 - 20) of the 9 patients that did not reconstitute significant numbers of sustained HLA-A24/pp65 specific $CD8^+$ T cells but were protected from repeated CMV replications are shown in Figure 3-10.

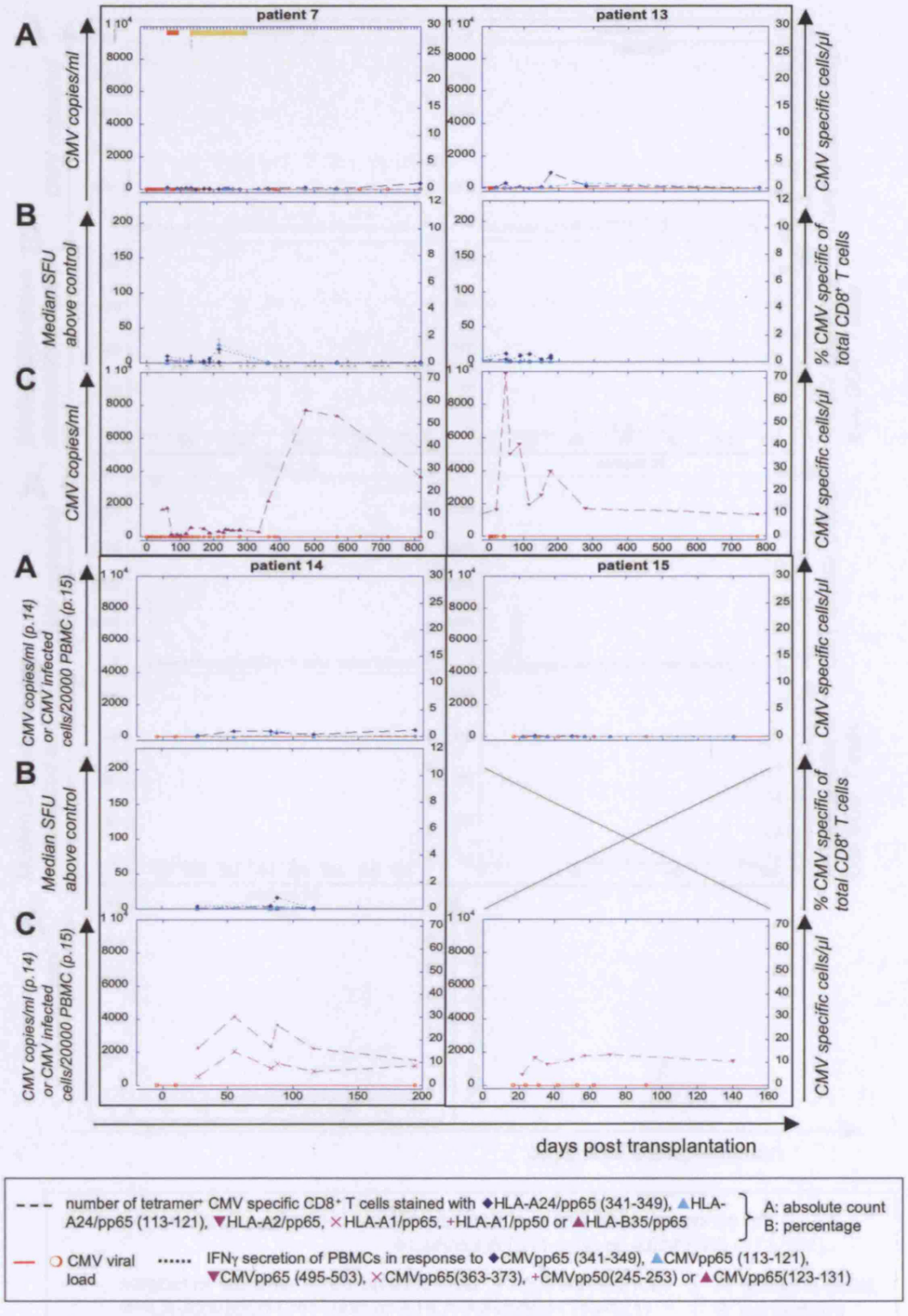


Figure 3-9 Monitoring of HLA-A24/pp65 specific CD8$^+$ T cell responses in patients 7 and 13 to 15

◆ HLA-A24/pp65(341-349) and ▲ HLA-A24/pp65(113-121) responses in A and B are shown in the same way as described for Figure 3-7 on page 198. An exception is patient 15, for whom the CMV viral load has been measured using antigenaemia with units expressed as infected cells per 200,000 PBMC. There have not been sufficient cell numbers to perform functional assays in this patient. Part C shows responses to other HLA/peptide targets (▼HLA-A2/pp65, ×HLA-A1/pp65, +HLA-A1/pp50, ▲HLA-B35/pp65). Horizontal bars indicate immunosuppressive (■ MMF: mycophenolate mofetil, ■ Prednisolone) treatment.

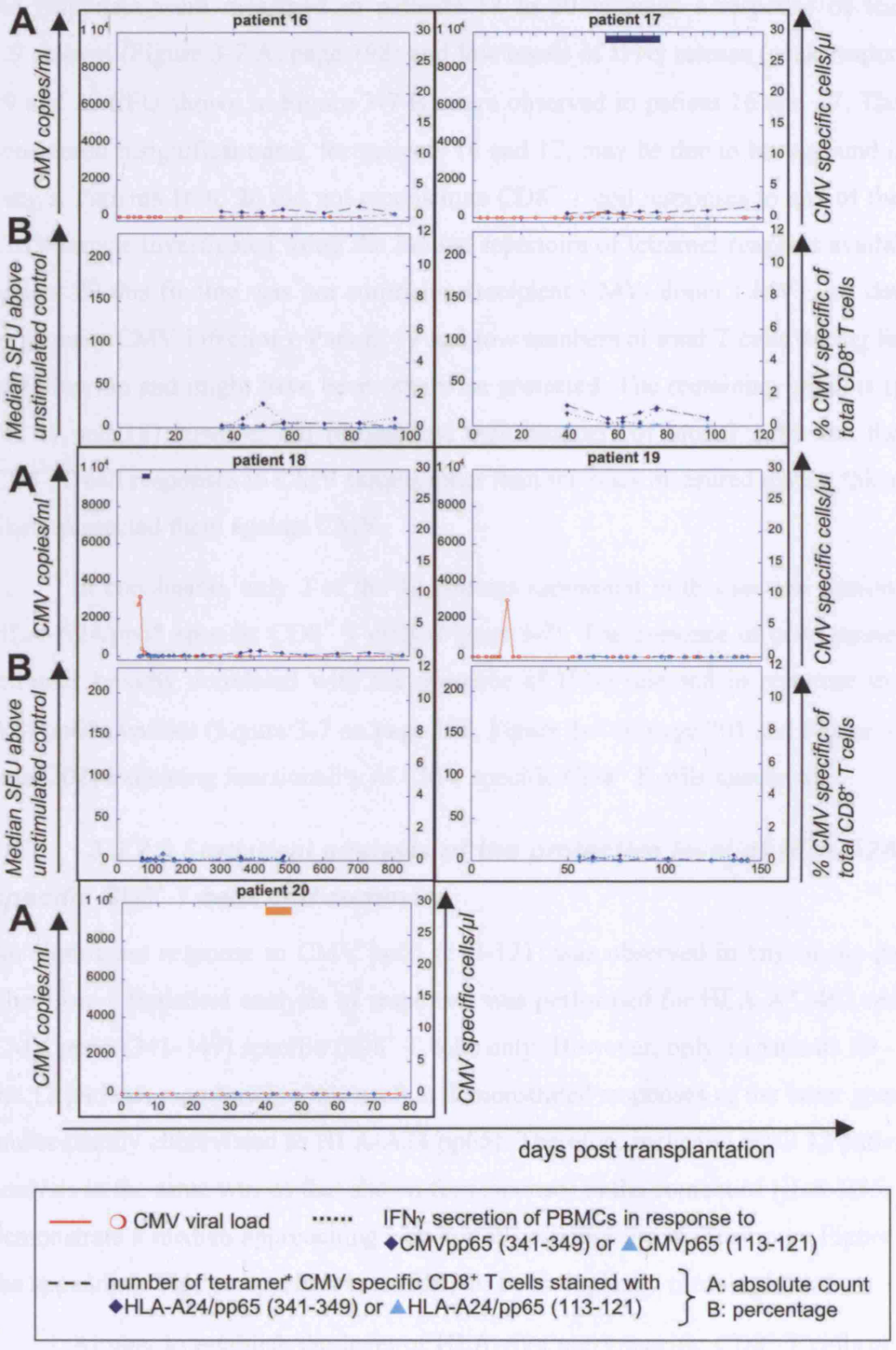


Figure 3-10 Monitoring of HLA-A24/pp65 specific CD8$^+$ T cell responses in patients 16 to 20

◆ HLA-A24/pp65(341-349) and ▲ HLA-A24/pp65(113-121) responses are shown in the same way as described for Figure 3-7 on page 198. Due to limited cell number available from patient 20, functional assays could not be performed. Horizontal bars in yellow/red or blue indicate immunosuppressive (■ Prednisolone) and antiviral (■ Ganciclovir) treatment respectively.

No responses were measured in patients 18 to 20 whereas a response of less than 1.9 cells/µl (Figure 3-7 A, page 198) and low levels of IFNγ release (peak responses of 29 and 20 SFU shown in Figure 3-7 B) were observed in patient 16 and 17. These are considered insignificant and, for patients 16 and 17, may be due to background in both assays. Patients 16 to 20 did not reconstitute $CD8^+$ T cell responses to any of the other CMV targets investigated using the limited repertoire of tetramer reagents available. In patient 20 this finding was not surprising (recipient CMV-/donor CMV+, no detection of primary CMV infection). Patient 19 had low numbers of total T cells during the time of follow-up and might have been otherwise protected. The remaining patients (patient 16, 17 and 18) however did reconstitute high numbers of total T cells and therefore $CD8^+$ T cell responses to CMV targets other than the ones measured during this project likely protected them against CMV.

In conclusion, only 3 of the 12 patients monitored in this section demonstrated HLA-A24/pp65 specific $CD8^+$ T cells (Figure 3-7). The presence of cells stained with tetramer broadly correlated with the presence of IFNγ released in response to HLA-A24/pp65 peptides (Figure 3-7 on page 198, Figure 3-9 on page 201 and Figure 3-10 on page 202) indicating functionality of CMV specific $CD8^+$ T cells measured.

3-2.2.3 Statistical analysis of the protective level of HLA-A24/pp65 specific $CD8^+$ T cells and summary

No significant response to CMV pp65 (113-121) was observed in any of the patients. Therefore a statistical analysis of responses was performed for HLA-A*2402 restricted CMV pp65 (341-349) specific $CD8^+$ T cells only. However, only 3 (patients 10 - 12) of the 12 patients monitored in this section demonstrated responses of the latter specificity (subsequently abbreviated as HLA-A24/pp65). Therefore inclusion of all 12 patients for analysis in the same way as that shown for responses in the context of HLA-B35, would demonstrate a median approaching zero for all response groups (compare Figure 6-4 in the appendix). This comparison is unlikely to be biologically meaningful.

Aiming to establish the level of HLA-A24/pp65 specific $CD8^+$ T cells at which patients were protected from CMV replication, measurements in patients that did not reconstitute these cells were omitted from the analysis. Responses in patients 10 to 12 (compare Figure 3-7 on page 198) were grouped depending on the occurrence of CMV replication at the time of sampling and a statistical comparison was performed in the same way as that shown for responses in the context of HLA-B35 (compare section 3-

2.1.4 from page 189 onwards). Among patients 10 to 12 there was none without CMV replication and therefore responses were assigned to two groups only, with results shown in Figure 3-11.

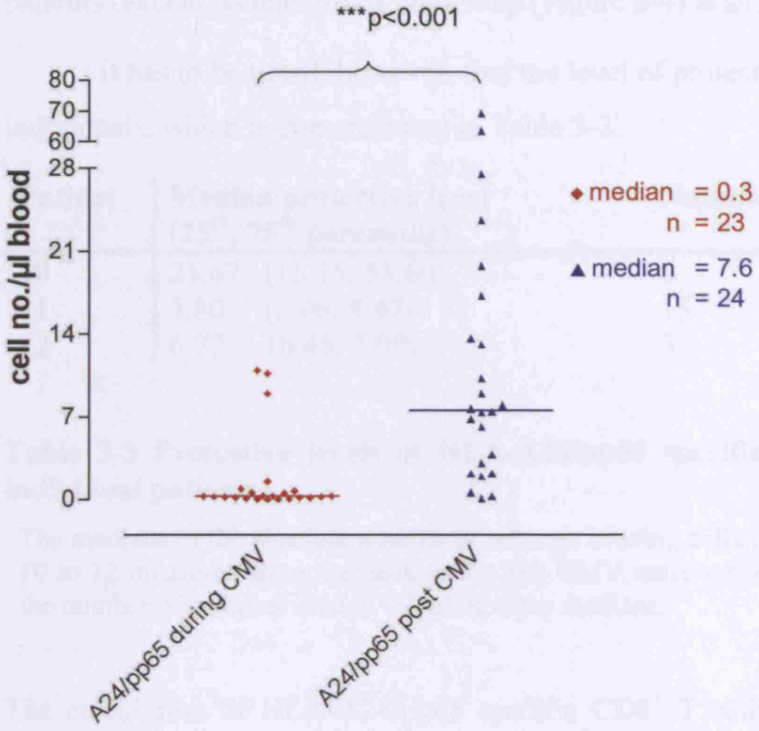


Figure 3-11 Statistical analysis of HLA-A24/pp65 (341-349) specific CD8$^+$ T cell responses

This figure illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in patients 10 (n= 11), 11 (n= 31) and 12 (n= 5) post transplantation until the last observed CMV reactivation (◆A24/pp65 during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in these patients (▲A24/pp65 post CMV). Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

Significantly higher responses were detected in patients following resolution of CMV reactivation (median 7.6 cells/µl) than during the period of CMV reactivation (median 0.3 cells/µl). The protective level of HLA-A24/pp65 specific CD8$^+$ T cells (7.6 cells/µl) will be compared to levels observed for CMV specific CD8$^+$ T cells targeting other HLA/peptide combinations in section 3-3 from page 238 onwards.

A possible limitation of this analysis would be that the "post CMV" group contained only those patients in whom HLA-A24/pp65 specific T cell levels were measurable; other patients were likely protected by other responses (responses from patient 20 were not included because the CMV seropositive patient received HSCT from a seronegative donor and primary infection was not detected) and therefore not informative. In the case of the "reactivating" group, if some patients never achieved

measurable levels of HLA-A24/pp65 specific T cells; these patients would be included since they clearly have no protective responses. However, there were none such patients in this group. For an objective comparison, a variation of this analysis including all patients (except patient 20) of this group (Figure 6-4) is shown in the appendix.

It has to be noted, however, that the level of protective responses varied between individuals, which is demonstrated in Table 3-3.

Patient	Median protective level (25^{th}, 75^{th} percentile)	Number of samples
10	21.67 (15.15, 53.60)	6
11	3.80 (2.06, 8.47)	15
12	6.77 (6.45, 7.09)	3

Table 3-3 Protective levels of HLA-A24/pp65 specific $CD8^+$ T cell responses in individual patients

The medians of the absolute number of tetramer binding cells/µl of peripheral blood in patients 10 to 12 measured from the peak of the last CMV reactivation onwards are listed along with the number of samples used to calculate these medians.

The calculation of HLA-A24/pp65 specific $CD8^+$ T cells correlating with protection from CMV reactivation resulted in a higher value obtained for patient 10 than for patients 11 and 12. However, following the initial peak of responses after the last CMV reactivation, cells declined to a level of 10.24 cells/µl at day 98 in patient 10, which is similar to an initial plateau level of 7.63 cells/µl at day 630 in patient 11 and 7.41 cells/µl at day 94 in patient 12 (Figure 3-7, page 198). HLA-A24/pp65 specific $CD8^+$ T cell levels in patient 10 only rose to higher levels after day 98. CMV reactivation was not observed after a level of 10.24 cells/µl was present at day 98 in patient 10. Subsequent high levels in that patient may therefore reflect temporary differences in homeostatic drive of T cells (refer to section 1-11.2, page 86 and section 3-3.3, page 244) between that patient and patients 11 and 12, which may be explained by their different transplant types (Table 2-1, page 99).

Low levels of HLA-A24/pp65 specific $CD8^+$ T cell responses were observed in healthy volunteers. Five CMV seropositive healthy volunteers expressing HLA-A*2402 were recruited and their blood tested for CMV pp65 (341-349) specific $CD8^+$ T cell responses. The frequencies of CMV specific $CD8^+$ T cells observed are shown in Table 3-4.

Healthy volunteer	HLA-A24/pp65 specific cells as a percentage of $CD8^+$ T cells	HLA-A24/pp65 specific cells per microlitre of blood
7	undetectable	undetectable
8	0.09	0.63
9	0.10	0.59
10	0.02	0.12
11	0.02	0.08

Table 3-4 HLA-A24/pp65 specific $CD8^+$ T cell levels in healthy volunteers

This table shows identification numbers of healthy volunteers along with the CMV pp65 (341-349) specific $CD8^+$ T cell counts detected in peripheral blood of these volunteers. Counts are represented as a percentage of the total $CD8^+$ T cell population and as a number per volume of blood.

They range from undetectable to 0.12 % of total $CD8^+$ T cells. This converts into a range of undetectable to 0.63 cells/µl of peripheral blood, which is lower than the level observed in HSCT patients ($p = 0.0022$) and might be considered as background.

3-2.3 CMV specific $CD8^+$ T cell responses restricted by HLA-A1

This section will demonstrate that the number of CMV specific $CD8^+$ T cells restricted by HLA-A1 that inversely correlated with the ability to detect CMV reactivation in HSCT patients are higher than those numbers of CMV specific $CD8^+$ T cells restricted by HLA-B35 or HLA-A24 that were shown in the previous sections.

Three CMV peptide targets studied in the context of HLA-A1 will be described first. This will be followed by results indicating that one of these targets does not induce $CD8^+$ T cell responses of significant frequency in HSCT patients. Longitudinal monitoring of $CD8^+$ T cells specific for the other two targets in individual patients will then show dominance of responses targeting the CMV pp50 epitope. Assignment of CMV pp50 specific $CD8^+$ T cells to different groups according to viral load in patients will be performed to establish the protective level of $CD8^+$ T cells to this target. Finally levels in patients and healthy volunteers will be summarised. A comparison to CMV specific $CD8^+$ T cell responses targeting other HLA/peptide combinations will be shown in section 3-3 from page 238 onwards.

3-2.3.1 CMV targets of $CD8^+$ T cell responses restricted by HLA-A1

Section 3-2.2 demonstrated $CD8^+$ T cell responses to the CMV pp65 (341-349) 9mer in the context of HLA-A*2402. During the time of this project it was reported that $CD8^+$ T cell responses to the similar CMV pp65 (341-350) 10mer occur in the context of HLA-A*2402 and HLA-A*0101 (Provenzano *et al.*, 2003). In that paper, $CD8^+$ T cell

responses to the 10mer in individuals expressing HLA-A*0101 or HLA-A*2402 were demonstrated by measurement of IFNγ mRNA expression of unmanipulated, peptide stimulated PBMC and by IFNγ protein production of PBMC in response to restimulation with the 10mer after 2 weeks *in vitro* sensitisation. The same group also described IFNγ mRNA and protein production of PBMC in response to restimulation with the 9mer pp65 (341-349) after 2 weeks *in vitro* sensitisation (Ghei *et al.*, 2005). It may therefore be possible to use $CD8^+$ T cell responses reactive to the (9mer or 10mer) peptide for adoptive therapy in patients, who express either the HLA-A*0101 or HLA-A*2402 alleles.

Therefore the 9mer QYDPVAALF and the 10mer QYDPVAALFF were tested against binding motifs and epitope prediction algorithms for HLA-A24 and HLA-A1 each within this study before attempting the generation of HLA-A1/pp65 (341-349) tetramer (for testing for the presence of HLA-A1 restricted pp65 (341-349) specific $CD8^+$ T cells within *ex vivo* PBMC in HSCT patients).

The amino acid sequence of QYDPVAALF(F) was compared to HLA motifs in the Syfpeithi (Rammensee *et al.*, 1999) and the HLA Ligand/Motif (Sathiamurthy *et al.*, 2003) databases. These are available at http://www.syfpeithi.de/ and http://hlaligand.ouhsc.edu respectively, comprising peptide sequences eluted from class I and class II MHC molecules together with entries compiled from published reports. Motifs defined for HLA-A*01 and HLA-A*2402 are shown in Table 3-5 and Table 3-6 below.

Peptide position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
		T	**D**				L		**Y**
		S	**E**						
		L		G	G	G			
				I	N	V			
				P	Y	I			
		T	*D*	*P*			*L*		*Y*
		S	*E*				*M*		
		M					*I*		
		I							

Table 3-5 HLA-A*01 peptide motifs

This table lists peptide motifs for HLA-A*01. Motifs shown in the upper part (Kubo *et al.*, 1994, DiBrino *et al.*, 1993, Falk *et al.*, 1994) were found in the Syfpeithi database and indicate anchor residues at the indicated position. Motifs shown in italics below the dashed line (DiBrino *et al.*, 1994, Kubo *et al.*, 1994) were found in the HLA ligand/motif database of the National Institutes of Health.

Peptide position	1	2	3	4	5	6	7	8	9
		Y							**L**
		F							**F**
									I
		Y							*F*
		F							*W*
									I
									L

Table 3-6 HLA-A*2402 peptide motifs

This table lists peptide motifs for HLA-A*2402. Motifs shown in the upper part (Maier *et al.*, 1994, Kubo *et al.*, 1994) indicate anchor residues at the indicated position that were found in the Syfpeithi database whereas motifs shown below the dashed line (Ibe *et al.*, 1996) were found in the HLA ligand/motif database of the National Institutes of Health.

Peptide QYDPVAALF(F) was found to fit the amino acid residue motifs for peptides binding to HLA-A*2402 (Table 3-6, Y at position 2 and F at position 9) and HLA-A*01 (Table 3-5, anchor D at position 3 and preferred residue P at position 4).

In addition to the comparison of peptide sequences with HLA binding motifs in the two databases described earlier, the peptide sequences were tested against epitope prediction algorithms built upon the Syfpeithi HLA ligand datasets (epitope prediction algorithms are not available from the HLA Ligand/Motif database). The score obtained during this test indicates the probability of the peptide of being processed and presented by the respective HLA allele. The scoring system evaluates every amino acid within a given peptide. Individual amino acids may be given the arbitrary value 1 for amino acids that are only slightly preferred in the respective position, optimal anchor residues are given the value 15; any value between these two is possible. Negative values are also possible for amino acids, which are disadvantageous for the peptide's binding capacity at a certain sequence position. The allocation of values is based on the frequency of the respective amino acid in natural ligands, T cell epitopes, or binding peptides (Rammensee *et al.*, 1999). For example, the maximal score for HLA-A*0201 peptides is 36. The well-known epitope GILGFVFTL derived from the influenza A matrix protein scores 30.

Testing of the QYDPVAALF(F) peptide sequence on Syfpeithi algorithms is shown in Table 3-7.

	9mer	10mer
HLA-A*2402	**24**	**21**
HLA-A*01	**12**	**14**

Table 3-7 Predicted score of ligation strength of QYDPVAALF(F) to HLA-A1 and HLA-A24

This table shows the ligation scores of the nonamer (9mer) QYDPVAALF and the decamer (10mer) QYDPVAALFF to HLA-A*2402 and HLA-A*01 according to the Syfpeithi algorithm (Rammensee *et al.*, 1999).

Section 3-2.2 (from page 194 onwards) showed investigations of the peptide QYDPVAALF in the context of HLA-A*2402. In contrast Figure 3-12 (page 210) in this section shows that this peptide could not be refolded with HLA-A*0101. Therefore 50 PBMC samples from CMV seropositive individuals bearing HLA-A*0101 but not HLA-A*2401 were tested for *ex vivo* IFNγ release of PBMC from HSCT patients in response to QYDPVAALF (Figure 3-13, page 212).

Along with responses targeting CMV pp65 (341-349), $CD8^+$ T cell responses targeting the well described 11mer located at the region pp65 363-373 with the sequence YSEHPTFTSQY (Longmate *et al.*, 2001, Retiere *et al.*, 2000) were investigated within this study. MHC class I molecules generally present peptides of 8 to 10 amino acids in size but longer peptides such as CMV pp65 (363-373) can also bind. Crystal structures demonstrated that longer peptides can maintain the conserved bonding networks around the N and C peptide termini, thereby displacing the centre of the peptide away from the groove to an elevated position above the α helices of the MHC (Probst-Kepper *et al.*, 2004, Speir *et al.*, 2001). Due to the likely bulge of CMV pp65 (363-373) it may fit not only the amino acid residues 2 and 3 (S and E at position 2 and 3) but also residue 9 (Y at position 11) of the motifs for peptides binding to HLA-A*01 (Table 3-5).

During the time of this project a new CMV peptide was reported to induce HLA-A*0101 restricted $CD8^+$ T cell responses (Elkington *et al.*, 2003). This peptide is a 9mer located at the region pp50 245-253 with the sequence VTEHDTLLY, which fits the amino acid residue motifs for peptides binding HLA-A*01 (Table 3-5, T at position 2, E at position 3, L at position 7 and Y at position 9). Publications before that reported that $CD8^+$ T cell responses to CMV are dominated by CMV pp65 (compare section 3-2.1.1, page 176). The study by Elkington and colleagues, however, described a significant response to the protein CMV pp50. In view of these new findings at the time, HLA-A*0101 restricted $CD8^+$ T cell responses to CMV pp50 (245-253) were

investigated along with responses to the two targets described above (CMV pp65 (341-349) and pp65(363-373)) and results are shown in this section.

3-2.3.2 Monitoring of CMV specific $CD8^+$ T cells in patients expressing HLA-A1

$CD8^+$ T cell responses to the peptide CMV pp65 (341-349) were assessed using the IFNγ ELISpot assay. Refolding of the peptide with HLA-A*0101 was not sufficient for generation of tetramer although the peptide could be refolded with HLA-A*2402. This is demonstrated in Figure 3-12. Therefore tetramer staining of CMV pp65 (341-349) specific $CD8^+$ T cell responses was not possible.

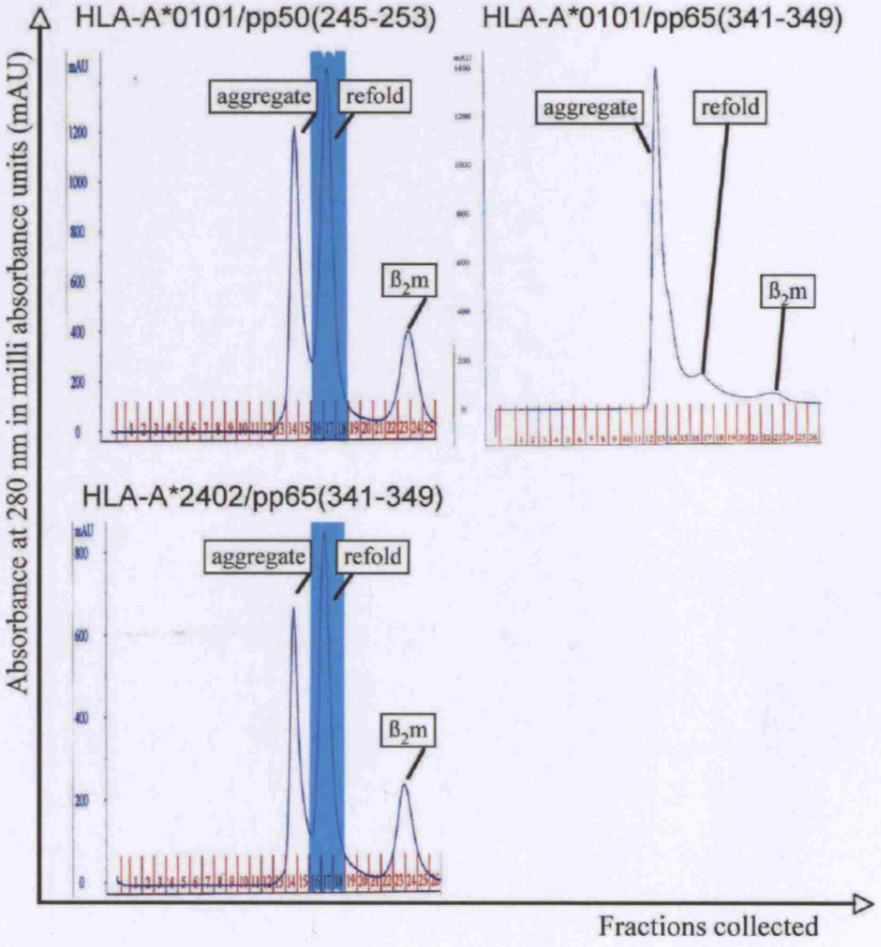


Figure 3-12 Refolding of HLA-A*0101/CMV pp65 (341-349)

This figure illustrates purification of refolded protein complex from aggregate, excess $ß_2m$ and excess peptide (compare Figure 2-20, page 153). FPLC traces on the left hand side demonstrate successful refolding of HLA-A*2402 with CMV pp65 (341-349) and successful refolding of HLA-A*0101 with another CMV peptide (marked in blue) whereas the FPLC trace on the right demonstrates very low efficiency of refolding of HLA-A*2402 with CMV pp65 (341-349).

50 samples were randomly selected from 10 patients and 1 healthy volunteer, who were CMV seropositive and expressed the HLA-A*0101 allele. They were used to test cytokine release from PBMC in response to stimulation with CMV pp65 (341-349) in comparison to pp50 (245-253) and pp65 (363-373) peptides that are known to be restricted by HLA-A*0101. Results can be seen in Figure 3-13.

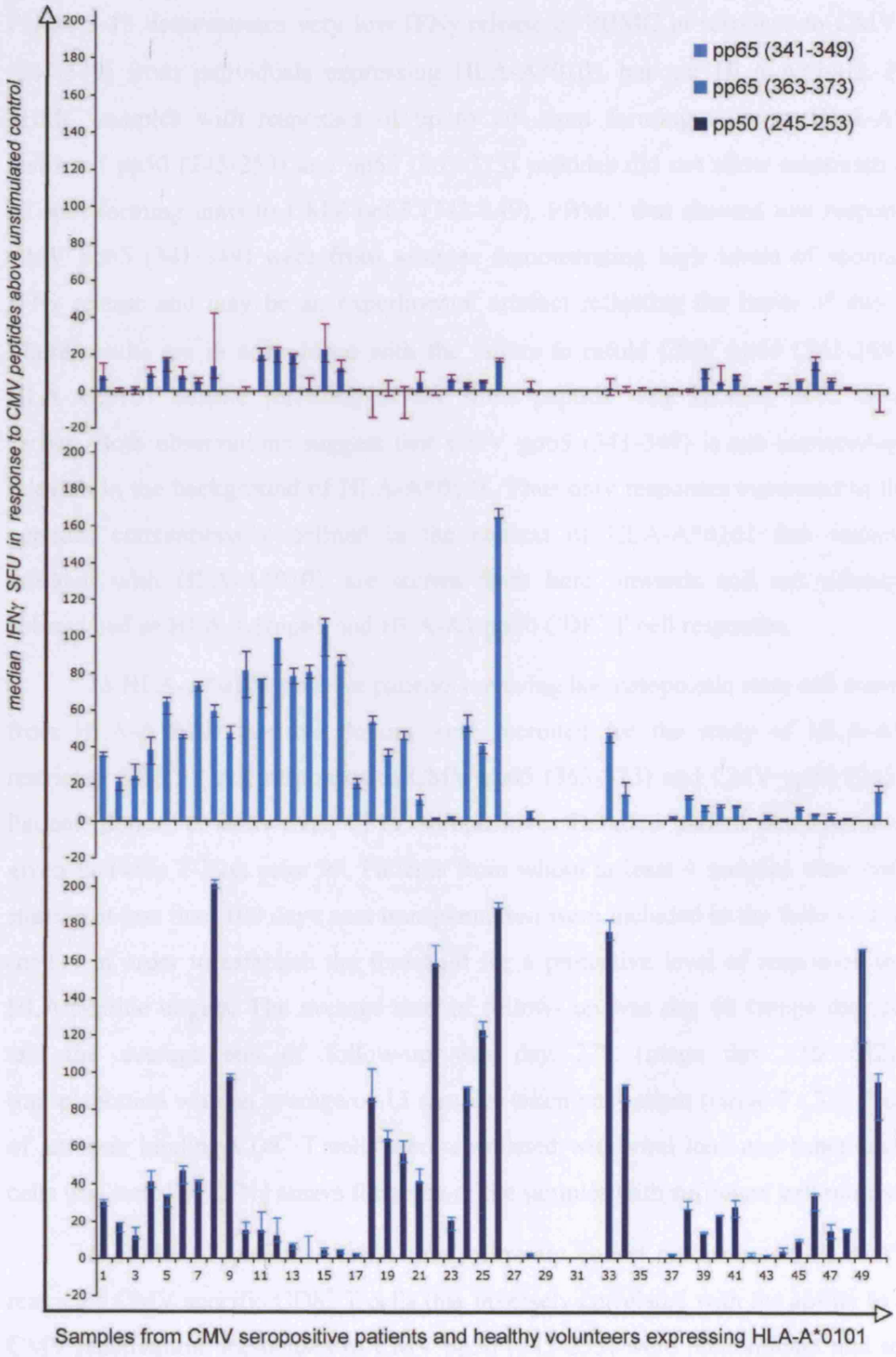


Figure 3-13 No significant IFNγ responses to CMV pp65 (341-349) but to CMV pp65 (363-373) and CMV pp50 (245-253) in individuals expressing HLA-A*0101

This diagram shows IFNγ responses to different peptides in 50 samples from CMV seropositive patients and one healthy volunteer expressing HLA-A*0101 but not HLA-A*2402. Responses to ■ pp65 (341-349) are shown on the top along with responses to ■ pp65 (363-373) in the middle and to ■ pp50 (245-253) on the bottom.

Figure 3-13 demonstrates very low IFNγ release of PBMC in response to CMV pp65 (341-349) from individuals expressing HLA-A*0101 but not HLA-A*2402. Patient PBMC samples with responses of up to 201 spot forming units to HLA-A*0101 restricted pp50 (245-253) and pp65 (363-373) peptides did not show responses above 22 spot forming units to CMV pp65 (341-349). PBMC that showed low responses to CMV pp65 (341-349) were from samples demonstrating high levels of spontaneous IFNγ release and may be an experimental artefact reflecting the limits of this assay. These results are in accordance with the failure to refold CMV pp65 (341-349) with HLA-A*0101 despite refolding of the same peptide with HLA-A*2402 described earlier. Both observations suggest that CMV pp65 (341-349) is not immunologically relevant in the background of HLA-A*0101. Thus only responses measured to the two peptides conventionally defined in the context of HLA-A*0101 that successfully refolded with HLA-A*0101 are shown from here onwards and are subsequently abbreviated as HLA-A1/pp65 and HLA-A1/pp50 $CD8^+$ T cell responses.

15 HLA-A*0101 positive patients receiving haematopoietic stem cell transplants from HLA-A*0101 positive donors were recruited for the study of HLA-A*0101 restricted $CD8^+$ T cell responses to CMV pp65 (363-373) and CMV pp50 (245-253). Patient, donor, or both were CMV seropositive. Detailed patient characteristics are given in Table 2-1 on page 99. Patients from whom at least 4 samples were collected starting at less than 100 days post transplantation were included in the follow-up cohort (n=11) in order to establish the threshold for a protective level of responses to these HLA/peptide targets. The average start of follow- up was day 48 (range day 20 - 90) and the average end of follow-up was day 279 (range day 136 - 682) post transplantation with an average of 13 samples taken per patient (range 5 - 33). Numbers of tetramer binding $CD8^+$ T cells were correlated with viral load and functionality of cells was tested by IFNγ assays for some of the samples with sufficient cell numbers.

5 of the 11 patients within this follow-up cohort reconstituted HLA-A*0101 restricted CMV specific $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation. Responses to CMV pp50 (245-253) were predominant and seemed protective in these patients whereas responses to CMV pp65 (363-373) were of lower frequency or absent. Figure 3-14 shows responses in 2 of these patients (patients 5 and 14) who did not reactivate CMV. Both patients appeared to be protected by HLA-A1/pp50 specific $CD8^+$ T cell responses. It should be noted that patient 5 additionally reconstituted HLA-B35/pp65 specific $CD8^+$ T cell responses (compare Figure 3-1, page

180). Patient 14 did not demonstrate protective responses in the context of other HLA types (compare Figure 3-9, page 201).

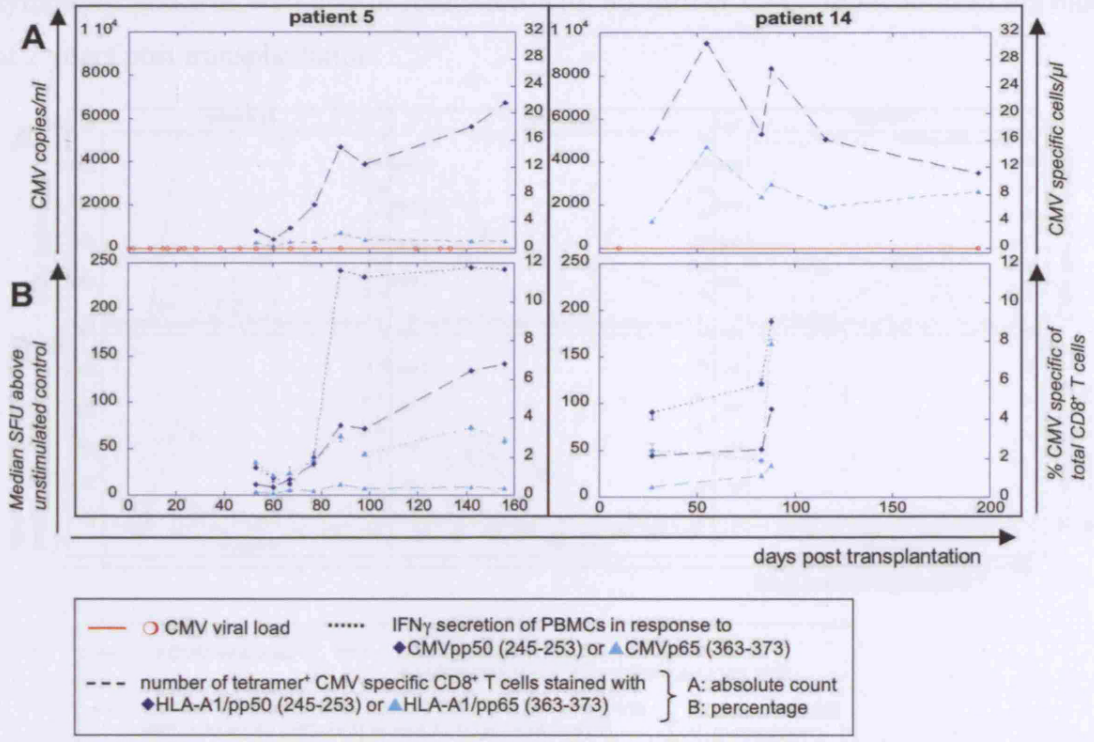


Figure 3-14 Monitoring of HLA-A*0101 restricted CMV specific CD8$^+$ T cell responses in patients 5 and 14

◆ HLA-A1/pp50(245-253) and ▲ HLA-A1/pp65(363-373) responses in two patients are shown in separate boxes. Viral load measurements are illustrated along with the absolute count of CMV specific CD8$^+$ T cell responses in A. Part B illustrates the IFNγ response to CMV peptide (triplicate measurements with the error representing the range from the 25th to the 75th percentiles) along with CMV specific CD8$^+$ T cell response as a percentage of the total CD8$^+$ T cell response (responses are only shown for time points when ELISpot analysis had been performed). Apart from standard prophylaxis described in section 2-2.5, patients did not receive any further immunosuppressive or antiviral treatment.

The other 3 of the 5 patients who reconstituted HLA-A1/pp50 specific CD8$^+$ T cell responses, initially reactivated CMV post HSCT and patient 23 additionally had CMV detected in his tissue samples. These patients received steroid treatment for GvHD and therefore had periods of suppressed T cell function. However, their CMV specific CD8$^+$ T cell numbers then increased, and subsequently these patients resolved their CMV reactivations with no further viral copies detected in their blood at the end of the follow-up period. These results are shown in Figure 3-15. Patient 21 received antiviral treatment only for his first CMV reactivation. Subsequent viral reactivations in this patient and reactivations observed in patient 22 were resolved without antiviral treatment. Patient 23 reconstituted high frequencies of HLA-A*0201 restricted CMV

specific CD8$^+$ T cell responses (Figure 3-21, page 229) in parallel with HLA-A*0101 restricted responses from day 185 post transplantation. He recovered from CMV related symptoms and was well and in remission with no further CMV replication in his blood at 2 years post transplantation.

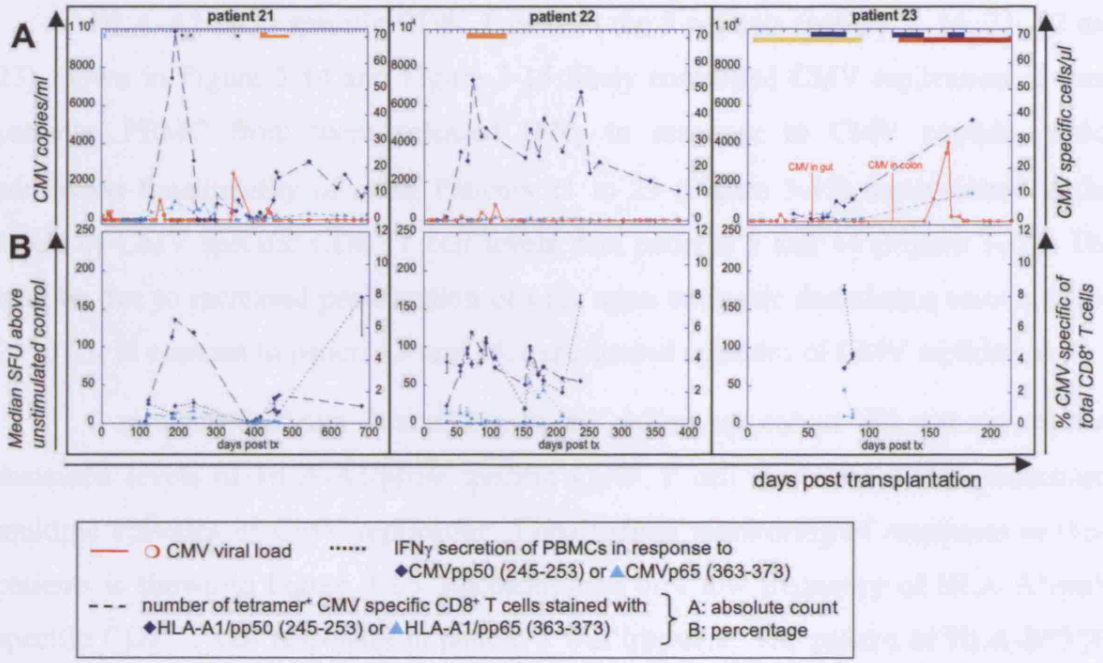


Figure 3-15 Monitoring of HLA-A*0101 restricted CMV specific CD8$^+$ T cell responses in patients 21 to 23

◆ HLA-A1/pp50(245-253) and ▲ HLA-A1/pp65(363-373) responses are shown in the same way as described for Figure 3-14 on page 214. Stars (*) indicate treatment using donor lymphocyte infusion (DLI). Horizontal yellow-red and blue bars indicate immunosuppressive (■ MMF, ■ Prednisolone, ■ Tacrolimus) and antiviral (■ Ganciclovir, ■ Foscarnet) treatment respectively.

Following the withdrawal of Cyclosporine on day 186 post transplantation patient 21 had become severely thrombocytopenic consistent with the recurrence of autoimmune antibodies associated with the recipient's FAS ligand deficiency. To establish full donor chimaerism that may eradicate the recipient B cell clones that are contributing to the immune phenomena, the patient received escalating doses of DLI. During this time HLA-A1/pp50 specific CD8$^+$ T cell numbers declined. GvHD since day 328 post transplantation was treated systemically with Prednisolone since day 419 post transplantation. This most likely caused HLA-A1/pp50 specific CD8$^+$ T cells to remain at low levels enabling further CMV replication. As the Prednisolone dose was reduced at day 437, HLA-A1/pp50 specific T cells were finally reconstituted and sustained without further reactivation. 3 years post transplantation the patient was in full chimaerism. It is assumed that a graft *versus* recipient B cell clone response occurred in

parallel with the GvH response. The patient demonstrated normal platelet counts, remained well and a case study has been published recently (Dimopoulou *et al.*, 2007) to report the first adult patient with ALPS (autoimmune lymphoproliferative syndrome) successfully being treated with HSCT and DLI.

HLA-A1/pp50 specific $CD8^+$ T cells in the 5 patients (patient 5, 14, 21, 22 and 23) shown in Figure 3-14 and Figure 3-15 likely controlled CMV replication in these patients. PBMC from them released IFNγ in response to CMV peptide, which confirmed functionality of cells. Patients 21 to 23 (Figure 3-15) reconstituted higher levels of CMV specific $CD8^+$ T cell levels than patients 5 and 14 (Figure 3-14). This may be due to increased proliferation of cells upon antigenic stimulation since patients 21 to 23, in contrast to patients 5 and 14, experienced episodes of CMV replication.

2 patients (patients 3 and 25) of the follow-up cohort did not reconstitute sustained levels of HLA-A1/pp50 specific $CD8^+$ T cell responses and demonstrated multiple episodes of CMV replication. Longitudinal monitoring of responses in these patients is shown in Figure 3-16. Reconstitution of a low frequency of HLA-A1/pp50 specific $CD8^+$ T cell responses in patient 3 was transient. The pattern of HLA-B*3501 restricted (Figure 3-1, page 180) and HLA-B*0702 restricted (not shown) CMV specific $CD8^+$ T cell responses in this patient closely resembled the pattern of responses shown here. All of these responses are of low frequency and correlate with very low numbers of total $CD8^+$ T cells (≤28 cells/μl except at days 470 and 491 post transplantation when 112 and 202 cells/μl were present respectively, cell numbers decreased again thereafter) in patient 3. Patient 25 also demonstrated low numbers of total $CD8^+$ T cells (≤61 cells/μl). Both patients were at risk for CMV reactivation and demonstrated multiple viral replication episodes accordingly.

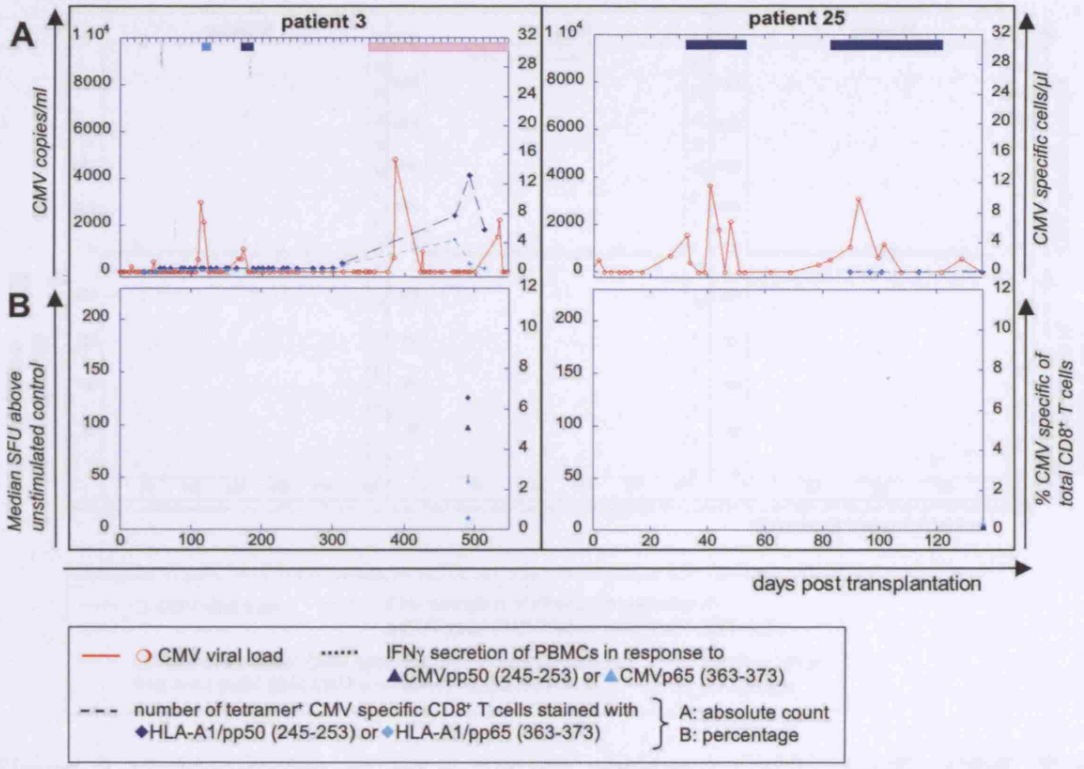


Figure 3-16 Monitoring of HLA-A*0101 restricted CMV specific CD8$^+$ T cell responses in patients 3 and 25

◆ HLA-A1/pp50(245-253) and ▲ HLA-A1/pp65(363-373) responses are shown in a similar way as described for Figure 3-14. Horizontal orange and blue bars indicate immunosuppressive (■ Dexamethasone) and antiviral (■ Ganciclovir, ■ Foscarnet) treatment respectively.

Responses from all of the 7 patients described above will be analysed statistically to establish the protective level of HLA-A1/pp50 specific CD8$^+$ T cells in section 3-2.3.3.

Similar to observations for HLA-A*2402 restricted CMV responses, there were also 3 patients (patients 26 - 28) in the cohort described here who did not have measurable CMV copies in their blood and did not reconstitute significant numbers of HLA-A*0101 CMV specific CD8$^+$ T cell responses. These data are shown in Figure 3-17 and Figure 3-18.

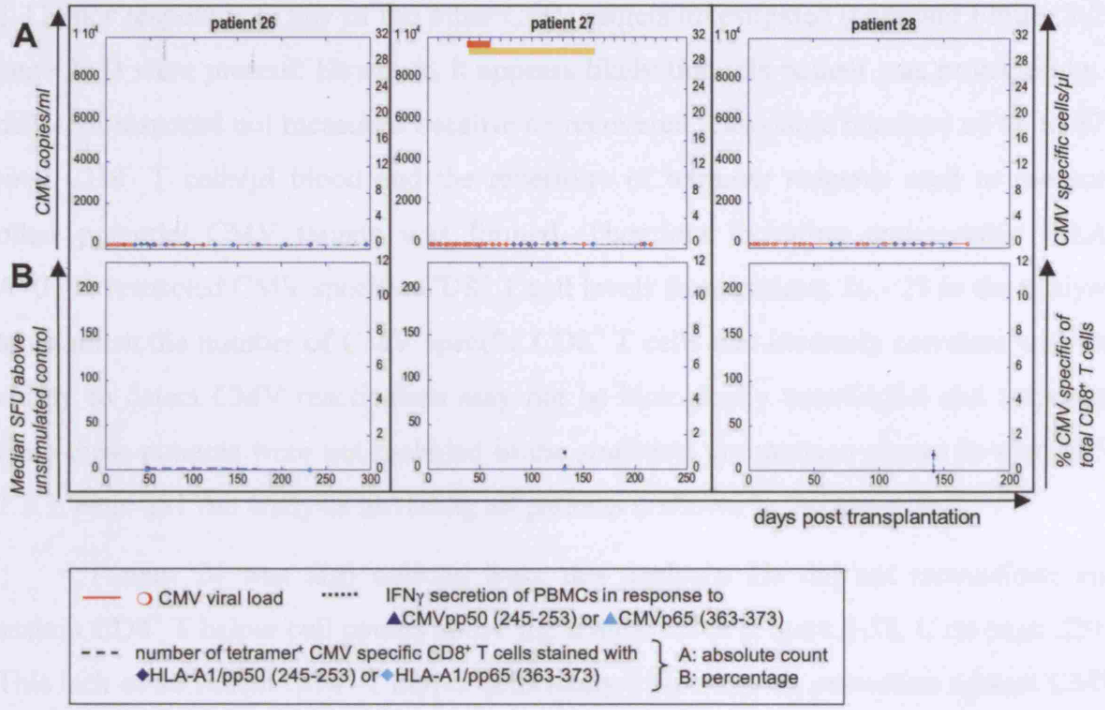


Figure 3-17 Monitoring of HLA-A*0101 restricted CMV specific CD8$^+$ T cell responses in patients 26 to 28

◆ HLA-A1/pp50(245-253) and ▲ HLA-A1/pp65(363-373) responses are shown in a similar way as described for Figure 3-14 on page 214. Horizontal yellow-red bars indicate immunosuppressive (■ MMF, ■ Prednisolone) treatment.

2 of these, patients 27 and 28, were CMV seronegative, received a transplant from a CMV seropositive donor and demonstrated no CMV reactivation. Previous studies suggest that expansion of CMV specific CD8$^+$ T cells post HSCT are likely antigen driven (Peggs *et al.*, 2003d) and therefore it is not expected that significant numbers of those cells would be detected in these patients. Interestingly, patient 28 did not reconstitute significant numbers of total CD8$^+$ T cells during the entire 5 months of the follow-up period post transplantation. DNA from PBMC collected at day 64, 113, 120 and 127 from this patient was therefore used in TREC analysis and results suggested that new CD8$^+$ T cell production had not occurred during that time period. Phenotypic analysis confirmed that the vast majority of T cells were CD4$^+$ and mainly of effector-memory phenotype (88.3 % and 87.1 % of CD4$^+$ T cells were CD27$^-$ and CD45RO$^+$ at day 113 and 127 post transplantation respectively). This is consistent with a failure of immune reconstitution.

The 3rd patient without significant numbers of HLA-A1/pp50 specific CD8$^+$ T cell responses and no CMV reactivation was patient 26, who was CMV seropositive and had a seropositive donor. No HLA-A1/pp50 specific CD8$^+$ T cell responses (Figure

3-17) nor responses to any of the other CMV targets investigated (compare Figure 3-23 page 231) were present. However, it appears likely that this patient was protected by a different response not measured because he recovered reasonable numbers of up to 477 total $CD8^+$ T cells/µl blood and the repertoire of tetramer reagents used to measure other potential CMV targets was limited. Therefore including undetectable HLA-A*0101 restricted CMV specific $CD8^+$ T cell levels from patients 26 - 28 in the analysis to establish the number of CMV specific $CD8^+$ T cells that inversely correlate with the ability to detect CMV reactivation may not be biologically meaningful and responses from these patients were not included in the statistical comparison shown in section 3-2.3.3, page 221 (an analysis including all patients is shown in the appendix).

Patient 24 was also omitted from this analysis. He did not reconstitute and sustain $CD4^+$ T helper cell counts above the critical value (Figure 3-18, C on page 220). This lack of sufficient $CD4^+$ T helper cells likely influences his protection against CMV as is discussed in more detail in section 3-3, page 238. It is likely that this underlies the observation that patient 24 undergoes CMV reactivation even while maintaining high levels of CMV specific $CD8^+$ T cells. This is demonstrated for HLA-A*0101 restricted $CD8^+$ T cells in Figure 3-18 below and for HLA-A*0201 restricted $CD8^+$ T cells in Figure 3-24 in section 3-2.4 on page 225.

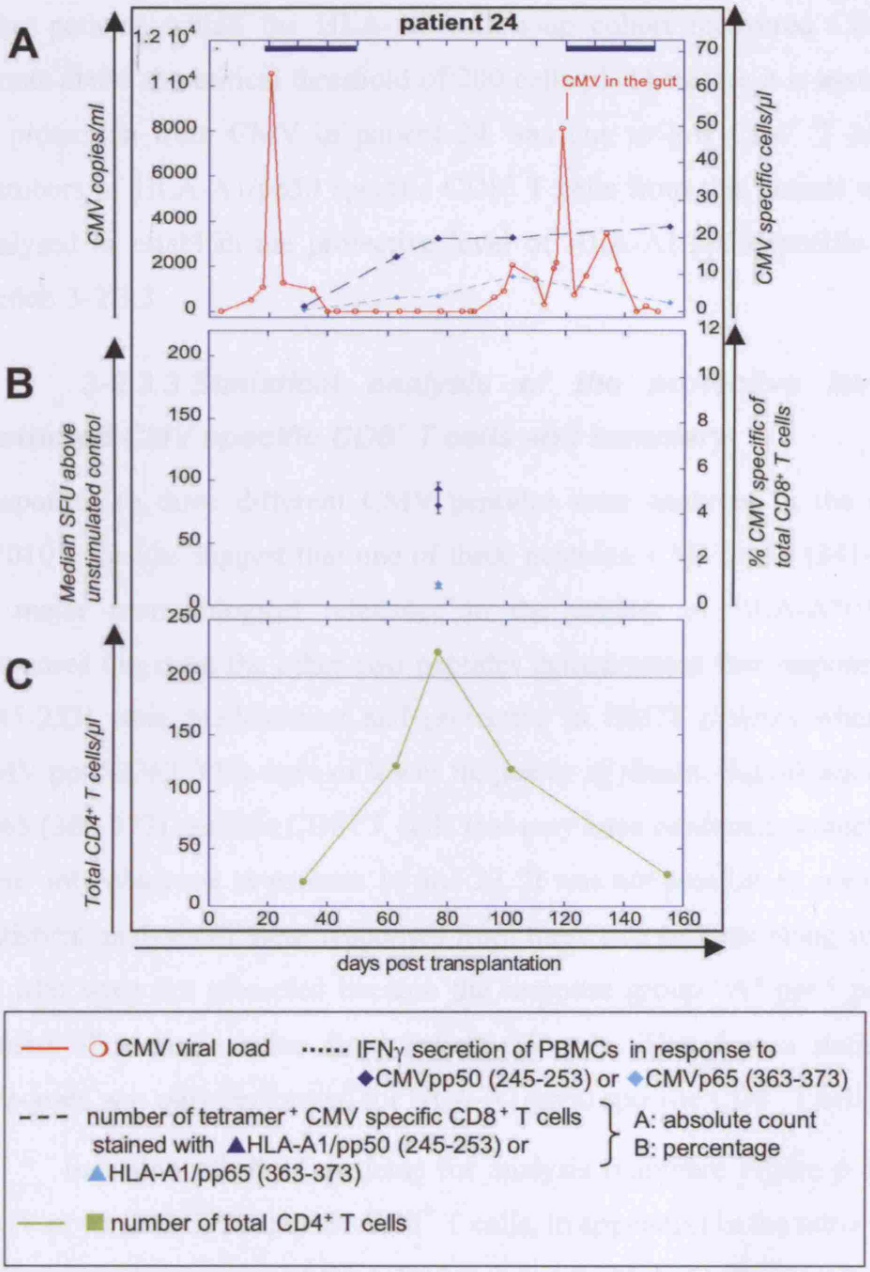


Figure 3-18 HLA-A*0101 restricted CMV specific CD8$^+$ T cell responses and lack of CD4$^+$ T cell help in patient 24

◆ HLA-A1/pp50(245-253) and ▲ HLA-A1/pp65(363-373) responses are shown in a similar way as described for Figure 3-14 (page 214) in A and B. CD4$^+$ T cells/µl of blood (●) are illustrated in graph C. The dotted line represents the critical value of CD4$^+$ T cells shown to correlate with infection and overall survival in HSCT patients (Kim *et al.*, 2006). Horizontal blue bars indicate antiviral treatment with Ganciclovir.

CD4$^+$ T helper cell counts ≥200 cells/µl were only temporarily reached at day 77 post transplantation in this patient. Before and after that time point CD4$^+$ T helper cell counts measured were below 123 cells/µl and CMV reactivations occurred. Finally the patient suffered from CMV disease and died. This is despite him having reconstituted high numbers of CMV pp50 (245-253) and some pp65 (363-373) specific CD8$^+$ T cells, which release IFNγ in response to the relevant peptide (Figure 3-18, page 220). All

other patients within the HLA-A1 follow-up cohort recovered $CD4^+$ T helper cell counts above the critical threshold of 200 cells/µl. Therefore it is assumed that the lack of protection from CMV in patient 24 was due to low $CD4^+$ T helper cell counts. Numbers of HLA-A1/pp50 specific $CD8^+$ T cells from this patient were therefore not analysed to establish the protective level of HLA-A1/pp50 specific $CD8^+$ T cells in section 3-2.3.3.

3-2.3.3 Statistical analysis of the protective level of HLA-A1 restricted CMV specific $CD8^+$ T cells and summary

Responses to three different CMV peptides were analysed in the context of HLA-A*0101. Results suggest that one of these peptides, CMV pp65 (341-349), may not be of major immunological relevance in the context of HLA-A*0101. Analysis of responses targeting the other two peptides demonstrated that responses to CMV pp50 (245-253) were predominant and protective in HSCT patients whereas responses to CMV pp65 (363-373) were of lower frequency or absent. Significant numbers of CMV pp65 (363-373) specific $CD8^+$ T cells that may have conferred protection against CMV were only observed in patients 14 and 23. It was not possible to perform a meaningful statistical analysis of these responses from these two patients along with patients 3 and 25 who were not protected because the response group "A1/pp65 post CMV" would consist of a single value from patient 23 only. Therefore a statistical analysis of responses was only performed for HLA-A1/pp50 specific $CD8^+$ T cells.

Inclusion of all 11 patients for analysis (compare Figure 6-5, which includes CMV pp65 (363-373) specific $CD8^+$ T cells, in appendix) in the same way as shown for responses in the context of HLA-B35 may not be biologically meaningful. 4 patients of this follow-up cohort were omitted from this analysis for reasons described in the previous section (3-2.3.2 from page 210).

Figure 3-19 shows a statistical analysis of the number of HLA-A1/pp50 tetramer binding $CD8^+$ T cells in correlation with viral load in patients 3, 5, 14, 21, 22, 23 and 25 (compare Figure 3-14 on page 214, Figure 3-15 on page 215 and Figure 3-16 on page 217).

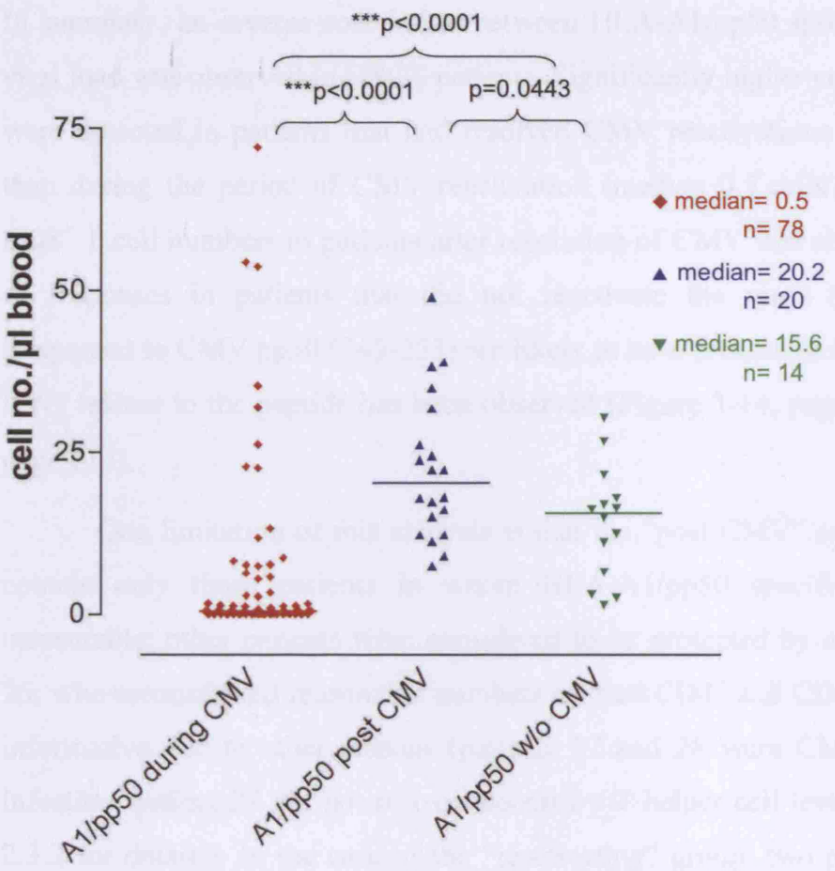


Figure 3-19 Statistical analysis of HLA-A1/pp50 specific CD8$^+$ T cell responses

This graph shows the absolute numbers of HLA-A1/pp50 tetramer binding cells/µl of peripheral blood in patients 3, 21, 22, 23 and 25 measured post transplantation until the last observed CMV reactivation (◆ during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in those patients that resolved their CMV reactivations (patients 21-23) (▲ post CMV) and absolute numbers of HLA-A1/pp50 tetramer binding cells in patients 5 and 14 who did not reactivate CMV (▼ w/o CMV). Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

The HLA-A1/pp50 specific CD8$^+$ T cell responses analysed in Figure 3-19 were detected at high levels during the CMV reactivation period in some of the samples from the CMV reactivating group of patients. One reason for this could be the initial reconstitution of protective levels of CMV specific T cells, which subsequently decreased and enabled further CMV reactivation. This can be observed in the pattern of T cell responses seen in patient 3 (Figure 3-16, page 217) and patient 21 (Figure 3-15, page 215). A temporary decrease in T cell levels was not observed for patients 22 and 23. Although blood samples were taken weekly in patient 22 at that time, it is possible that a decrease in T cell levels was missed in between samples taken from patient 22 and especially patient 23 as the clinical status of the latter prohibited frequent sampling at the time before his last CMV reactivation period (Figure 3-15, page 215).

In summary, an inverse correlation between HLA-A1/pp50 specific $CD8^+$ T cells and viral load was observed in HSCT patients. Significantly higher cell numbers ($p<0.0001$) were detected in patients that had resolved CMV reactivations (median 20.2 cells/µl) than during the period of CMV reactivation (median 0.5 cells/µl). The magnitude of $CD8^+$ T cell numbers in patients after resolution of CMV was similar to the magnitude of responses in patients that did not reactivate the virus (median 15.6 cells/µl). Responses to CMV pp50 (245-253) are likely to have protective capacity in patients and IFNγ release to the peptide has been observed (Figure 3-14, page 214 and Figure 3-15, page 215).

One limitation of this analysis is that the "post CMV" and "w/o CMV" groups contain only those patients in whom HLA-A1/pp50 specific T cell levels were measurable; other patients were considered to be protected by other responses (patient 26, who reconstituted reasonable numbers of total $CD4^+$ and $CD8^+$ T cells) or to be not informative due to other reasons (patients 27 and 28 were CMV-/+ without primary infection, patient 24 did not recover necessary T helper cell levels, compare section 3-2.3.2 for details). In the case of the "reactivating" group, two patients (patients 3 and 25) never achieved measurable levels of HLA-A1/pp50 specific T cells; these patients are included since they clearly have no protective responses. Their total $CD8^+$ T cell numbers are very low and it is impossible to determine what protective responses they may achieve in the future, if any.

For an objective comparison, variations of this analysis that either include all patients (except patients 27 and 28 who were CMV seronegative and received HSCT from a seropositive donor, after which primary infection was not detected) of this group (Figure 6-5) or exclude patients 3 and 25 (Figure 6-6) are shown in the appendix. The latter demonstrates significant differences between response groups comparable to Figure 3-19.

The number of HLA-A1/pp50 specific $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation in HSCT patients and levels observed in healthy volunteers (as described below) will be compared to levels observed for CMV specific $CD8^+$ T cells targeting other HLA/peptide combinations in section 3-3 from page 238 onwards. The level of protective responses varied between individual patients, which is demonstrated in Table 3-8.

Patient	Median protective level (25^{th} percentile, 75^{th} percentile)	Number of samples
5	9.47 (2.93, 15.71)	8
14	16.67 (16.22, 24.29)	6
21	14.20 (11.46, 17.51)	8
22	24.46 (20.16, 33.38)	11
23	*38.24* -	*1*

Table 3-8 Protective levels of HLA-A1/pp50 specific $CD8^+$ T cell responses in individual patients

The median values of all absolute numbers of tetramer binding cells/µl of peripheral blood measured in patients 5 and 14 (who did not reactivate CMV) and of values measured from the peak of the last CMV reactivation onwards in patients 21, 22 and 23 (in italics: calculated from <3 samples) are illustrated along with the number of samples used to calculate each median value.

The calculation of HLA-A1/pp50 specific $CD8^+$ T cells correlating with protection from CMV reactivation resulted in a higher level obtained for patient 23 than for other patients. However, the level for patient 23 was calculated from a single value only (Figure 3-15 on page 215), which may reflect the peak of the response.

CMV specific T cell levels measured in 5 CMV seropositive volunteers expressing HLA-A*0101 are shown in (Table 3-9).

Healthy volunteer	HLA-A1 restricted cells as a percentage of $CD8^+$ T cells		HLA-A1 restricted cells per microlitre of blood	
	CMV pp50	CMV pp65	CMV pp50	CMV pp65
1	0.28	0.04	1.00	0.16
3	0.43	0.07	2.49	0.41
12	0.92	0.93	3.00	3.03
13	3.58	0.05	14.32	0.20
14	0.79	0.58	3.16	2.32

Table 3-9 HLA-A*0101 restricted CMV pp65 (363-373) and CMV pp50 (245-253) specific $CD8^+$ T cell levels in healthy volunteers

This table shows CMV specific $CD8^+$ T cell counts detected in peripheral blood of CMV seropositive healthy volunteers expressing HLA-A*0101. Counts are represented as a percentage of the total $CD8^+$ T cell population and as a number per volume of blood.

The results observed in healthy volunteers show that - similar to observations in HSCT patients - the level of responses to CMV pp50 (245-253) were equal to (volunteer 12 and 14) or greater than (volunteer 1, 3 and 13) responses to CMV pp65 (363-373) in the same HLA-A*0101 expressing CMV seropositive individuals.

HLA-A1/pp50 specific $CD8^+$ T cell numbers range from 0.28 to 3.58 % of total $CD8^+$ T cells. This converts into a range of 1.00 to 14.32 cells/µl of peripheral blood, which is lower than the level observed in HSCT patients ($p = 0.0046$), compare Figure 3-26 on page 241.

A comparison of the levels described here with the levels of CMV specific $CD8^+$ T cells targeting other HLA/peptide combinations will be shown in section 3-3 from page 238 onwards.

3-2.4 CMV specific $CD8^+$ T cell responses restricted by HLA-A2

In the sections above measurements of protective levels of responses towards different HLA/CMV peptide combinations were established for comparison with measurements of HLA-A*0201 restricted CMV pp65 (495-503) specific $CD8^+$ T cells. Most of the knowledge about CMV specific $CD8^+$ T cells has been derived from studying cells targeting the latter HLA/peptide combination. Due to the high prevalence of the HLA-A*0201 allele in the Caucasoid population, HLA-A*0201 restricted CMV pp65 (495-503) specific $CD8^+$ T cells are one of the most studied CMV specific $CD8^+$ T cells and as such might be considered as a archetype for comparison of CMV specific $CD8^+$ T cell responses targeting other HLA/peptide combinations. To make the best possible direct comparison between responses to new targets studied in this project and responses to the archetype target, HLA-A*0201 restricted CMV pp65 (495-503) specific $CD8^+$ T cell responses were studied and are shown in this section.

This allowed for not only comparing responses towards new HLA/CMV peptide targets with HLA-A*0201 restricted CMV specific responses that were established in previous studies but also comparing those responses with the level of HLA-A*0201 restricted CMV specific responses measured in this study. This comparison will be shown in section 3-3 from page 238 onwards.

Patients and volunteers recruited for analysis in this section expressed HLA-A*0201 but not HLA-B*0702 because it is known that CMV specific $CD8^+$ T cell responses restricted by HLA-B*0702 dominate those restricted by HLA-A*0201 (Lacey *et al.*, 2003). This observation is also discussed further in section 3-3.

3-2.4.1 CMV targets of CD8$^+$ T cell responses restricted by HLA-A2

$CD8^+$ T cells that were investigated in this section targeted the CMV pp65 (495-503) peptide with the sequence NLVPMVATV, which was originally described by Wills and colleagues (Wills *et al.*, 1996). HLA-A*0201 restricted CMV pp65 (495-503) specific $CD8^+$ T cell responses are subsequently abbreviated as HLA-A2/pp65 specific $CD8^+$ T cell responses.

3-2.4.2 Monitoring of CMV specific CD8$^+$ T ceils in patients expressing HLA-A2

15 HSCT patients bearing the HLA-A*0201 allele who received a transplant from HLA-A*0201 positive donors were recruited for the study of HLA-A2/pp65 specific $CD8^+$ T cell responses. Patient, donor, or both were CMV seropositive with detailed patient characteristics shown in Table 2-1 on page 99. Patients from whom at least 4 samples were collected starting at less than 100 days post transplantation were included in the follow-up cohort (n = 10) in order to establish the threshold for a protective level of responses to this HLA/peptide target. The average start of follow- up was day 36 (range day 11 - 88) and the average end of follow-up was day 368 (range day 145 - 902) post transplantation with an average of 15 samples taken per patient (range 5 - 39). Numbers of tetramer binding $CD8^+$ T cells were correlated with viral load and the functionality of cells was tested by IFNγ assays for some of the samples with sufficient cell numbers.

4 of the 10 patients within this follow-up cohort reconstituted significant numbers of HLA-A2/pp65 specific $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation (Figure 3-20 and Figure 3-21) and will be discussed first. Data on the remaining 6 patients will be described thereafter.

Figure 3-20 shows the responses in 2 patients (patients 13 and 15) who reconstituted and sustained HLA-A2/pp65 specific $CD8^+$ T cells at a similar plateau level and were protected from CMV replication. Due to a history of CMV pneumonitis, patient 13 received high doses of prophylactic antiviral treatment until day 97 post transplantation, which may have helped to prevent CMV reactivation in the early phase post transplantation. Both patients also expressed HLA-A*2402 but did not reconstitute CMV specific $CD8^+$ T cells restricted by that HLA type (Figure 3-9, page 201). HLA-A2/pp65 specific $CD8^+$ T cell responses were assumed to be protective in both individuals.

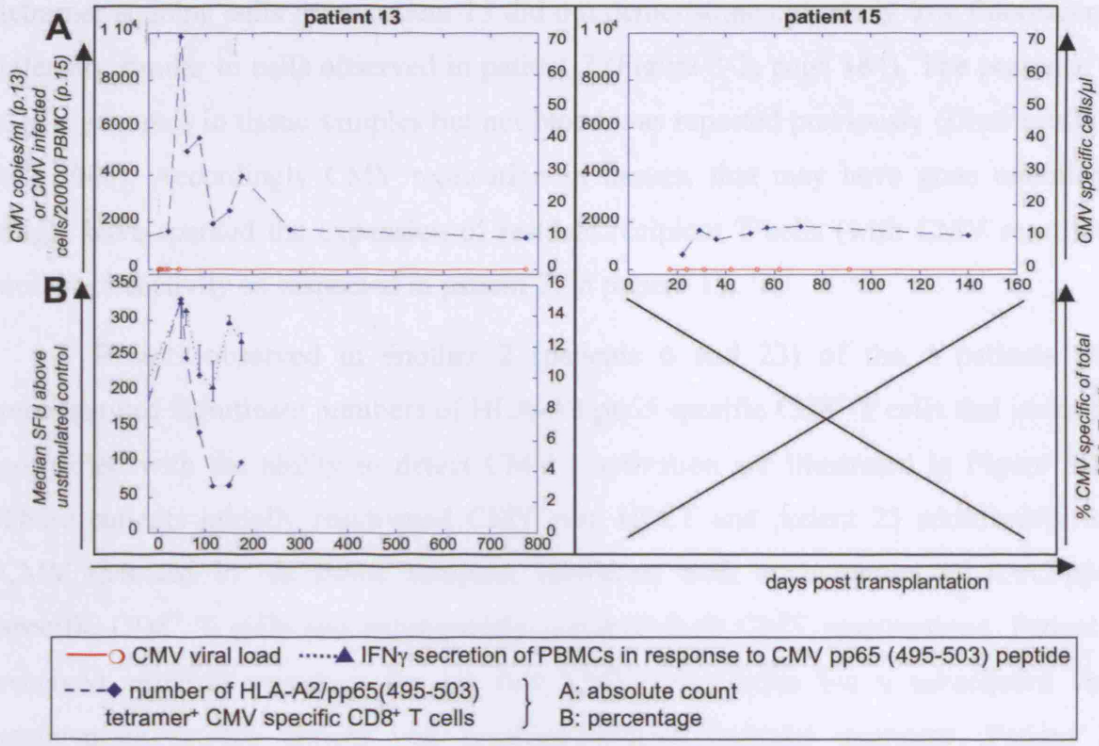


Figure 3-20 Monitoring of HLA-A2/pp65 specific CD8$^+$ T cell responses in patients 13 and 15

HLA-A2/pp65 specific CD8$^+$ T cells are shown. Part A represents viral load measurements along with the absolute count of CMV specific CD8$^+$ T cells. In patient 15 CMV viral load has been measured using antigenaemia instead of PCR with units expressed as infected cells per 200,000 PBMC. Part B represents the IFNγ response to CMV peptide (triplicate measurements with the error representing the range from the 25th to the 75th percentiles) along with CMV specific CD8$^+$ T cells as % of total CD8$^+$ T cells (responses are only shown for time points when ELISpot analysis had been performed). ELISpot assays were not performed on samples from patient 15 due to insufficient cell numbers available. Apart from standard prophylaxis (described in section 2-2.5) patients did not receive any further immunosuppressive or antiviral treatment.

At first sight, the development of an early CMV specific response in patient 15 was surprising. Like patient 7 described in section 3-2.1.3 (page 182), this patient was CMV seropositive and had received a non-TCD transplant from a CMV seronegative donor. During conventional transplantation, recipient T cells typically survive only when the graft is thoroughly depleted of donor T cells (Roux *et al.*, 1992). However, (in contrast to patient 7) patient 15 had received reduced-intensity conditioning (RIC) before HSCT (Table 2-1, page 99). Thereby mixed chimaerism with 45 - 55 % donor cells was established in the patient during the time of follow-up. This might explain the persistence of recipient T cells in this patient. In comparison, earlier throughout this chapter (section 3-2.1.3, page 182) it was hypothesised that cells from patient 7 (receiving myeloablative conditioning) may be crossreactive to alloantigen. It appears, however, that this is not applicable for patient 15, who did not experience GvHD. Also,

tetramer staining cells from patient 15 did not demonstrate extremely low fluorescence intensity similar to cells observed in patient 7 (Figure 3-2, page 184). The presence of CMV genomes in tissue samples but not blood was reported previously (Dimitroulia *et al.*, 2006). Accordingly CMV replication in tissues, that may have gone unnoticed, might have sparked the expansion of residual recipient T cells (with CMV reactivity, not crossreactivity as suspected in patient 7) in patient 15.

Results observed in another 2 (patients 6 and 23) of the 4 patients who reconstituted significant numbers of HLA-A2/pp65 specific $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation are illustrated in Figure 3-21. These patients initially reactivated CMV post HSCT and patient 23 additionally had CMV detected in his tissue samples. However, both reconstituted HLA-A2/pp65 specific $CD8^+$ T cells and subsequently resolved their CMV reactivations. Patient 6 received antiviral treatment for his first CMV reactivation but a subsequent viral reactivation in this patient was resolved without antiviral treatment. Patient 23 reconstituted high frequencies of HLA-A2/pp65 specific $CD8^+$ T cells from day 185 post transplantation. He recovered from CMV related symptoms and was well and in remission with no further CMV copies in his blood at 2 years post HSCT. Viral copies remained undetected after the end of the follow-up period in both patients suggesting that the measured T cell responses were protective. Patient 6 also reconstituted HLA-B35/pp65 specific $CD8^+$ T cells (Figure 3-1, page 180) and patient 23 also reconstituted HLA-A1/pp50 specific $CD8^+$ T cells (Figure 3-15, page 215).

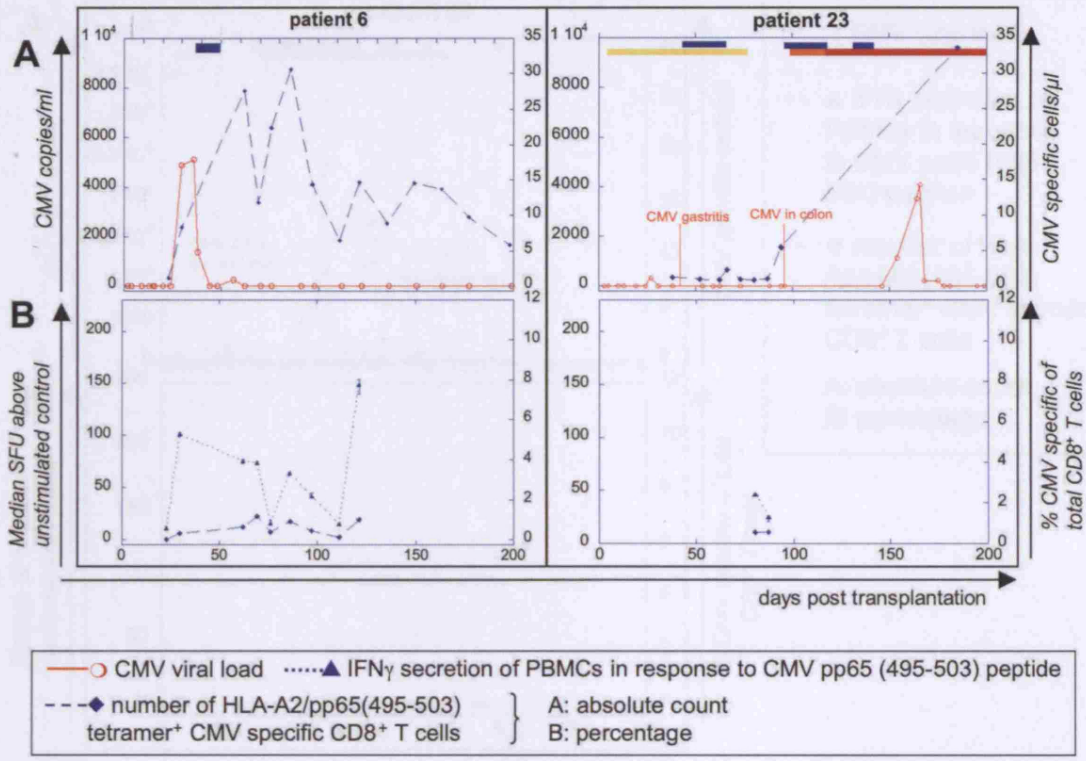


Figure 3-21 Monitoring of HLA-A2/pp65 specific CD8$^+$ T cell responses in patients 6 and 23

HLA-A2/pp65 specific CD8$^+$ T cell responses of two patients are shown in the same way as described for Figure 3-20 on page 227. Horizontal yellow-red and blue bars indicate immunosuppressive (■ MMF, ■ Prednisolone, ■ Tacrolimus) and antiviral (■ Ganciclovir) treatment respectively.

HLA-A2/pp65 specific CD8$^+$ T cells in the 4 patients shown in Figure 3-20 (page 227) and Figure 3-21 likely controlled CMV replication in these patients. PBMC from these patients released IFNγ in response to CMV peptide, which confirmed the functionality of these cells.

One patient (patient 30) of this follow-up cohort did not reconstitute sustained levels of HLA-A*0201 restricted CD8$^+$ T cell responses to CMV pp65 (495-503) and experienced multiple episodes of CMV replication despite antiviral treatment. Longitudinal monitoring of CMV specific CD8$^+$ T cell numbers in this patient is shown in Figure 3-22.

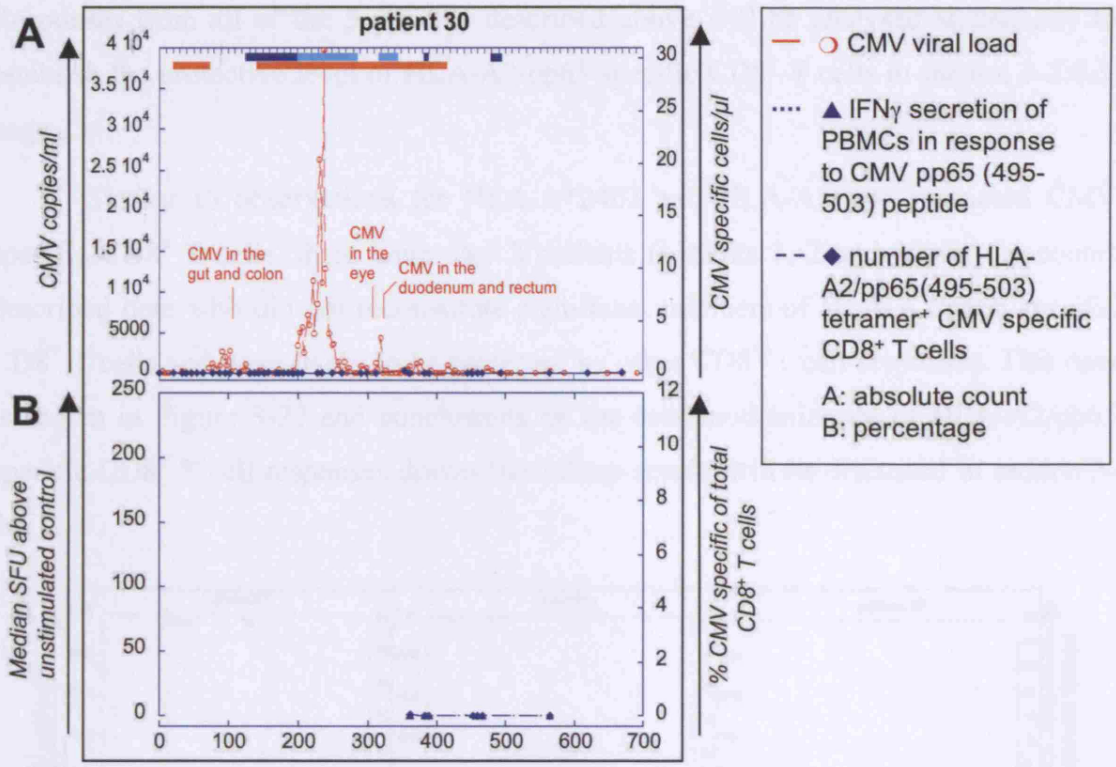


Figure 3-22 Monitoring of HLA-A2/pp65 specific CD8$^+$ T cell responses in patient 30

HLA-A2/pp65 specific CD8$^+$ T cell responses are shown in the same way as described for Figure 3-20 on page 227. Horizontal yellow-red and blue bars indicate immunosuppressive (■ Prednisolone,) and antiviral (■ Ganciclovir or Valganciclovir, ■ Foscarnet) treatment respectively.

The donor of patient 30 was CMV seronegative. Conditioning with Campath-1H as well as prolonged immunosuppressive treatment that was received by patient 30 post transplantation due to acute and chronic GvHD may have hindered the development of CMV specific T cell responses of a level adequate for protection. This patient received prophylactic cyclosporine A during the entire period of the follow-up. HLA-A2/pp65 specific CD8$^+$ T cell responses were absent post allogeneic transplantation and the patient developed CMV disease involving the gastrointestinal tract and also leading to blindness in one eye. Total numbers of CD4$^+$ and CD8$^+$ T cells were first recovered at day 350 post HSCT and remained at high levels from day 564 onwards. Interestingly, this patient previously had an autologous transplant, after which high levels of nearly 1x10^8 HLA-A2/pp65 specific CD8$^+$ T cells/L of blood could be observed (data not shown, samples from this time period are referred to as from patient 30b throughout this thesis).

Responses from all of the 5 patients described above will be analysed statistically to establish the protective level of HLA-A2/pp65 specific $CD8^+$ T cells in section 3-2.4.3, page 234.

Similar to observations for HLA-A*2402 and HLA-A*0101 restricted CMV specific $CD8^+$ T cells, there were also 3 patients (patients 1, 2 and 26) in the cohort described here who did not reconstitute significant numbers of HLA-A2/pp65 specific $CD8^+$ T cells and were likely to be protected by other $CD8^+$ T cell responses. This data is shown in Figure 3-23 and conclusions on the immunodominance of HLA-A2/pp65 specific $CD8^+$ T cell responses drawn from these results will be discussed in section 3-2.4.3.

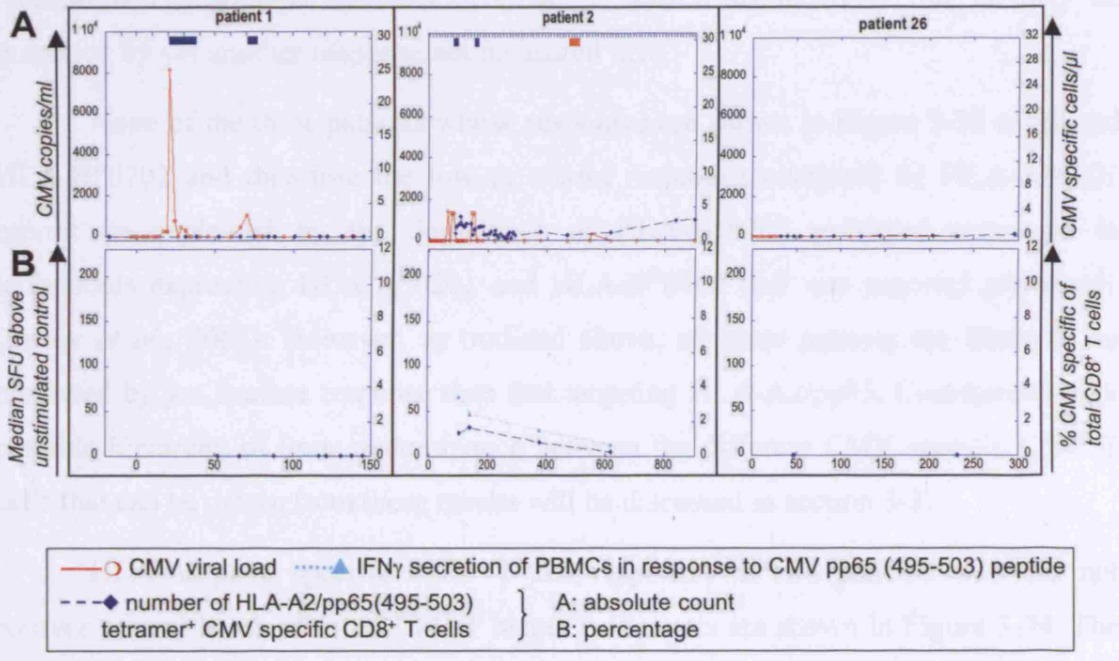


Figure 3-23 Monitoring of HLA-A2/pp65 specific $CD8^+$ T cell responses in patients 1, 2 and 26

HLA-A2/pp65 specific $CD8^+$ T cell responses are shown in a similar way as described for Figure 3-20 on page 227. Horizontal yellow-red and blue bars indicate immunosuppressive (■ Prednisolone,) and antiviral (■ Ganciclovir) treatment respectively.

No HLA-A2/pp65 specific $CD8^+$ T cells were detected in patient 1. Nevertheless two reactivations of CMV were quickly resolved. One contributing factor may be the antiviral treatment, which is indicated in the graph. However, the patient did reconstitute HLA-B35/pp65 specific $CD8^+$ T cells (Figure 3-1 on page 180). Therefore those cells may have been protective in this patient.

Patient 2 also reconstituted HLA-B35/pp65 specific $CD8^+$ T cells (Figure 3-1, page 180) in parallel to HLA-A2/pp65 specific $CD8^+$ T cells (Figure 3-23). The levels of both responses were similar initially but after the last CMV reactivation at day 549 post transplantation responses restricted by HLA-A2 were lower than those restricted by HLA-B35. This situation is not easy to interpret. It seems most likely that CMV specific $CD8^+$ T cell responses restricted by HLA-B35 were protective in the patient whereas responses restricted by HLA-A2 were a bystander effect because the frequency of the latter was near the background level observed for the relevant tetramer.

Despite a lack of detectable HLA-A2/pp65 specific $CD8^+$ T cells in patient 26, this patient did not reactivate CMV (Figure 3-17, page 218). Given that he had reconstituted reasonable numbers of $CD8^+$ T cells it seems likely that he may be protected by yet another response not measured here.

None of the three patients whose responses are shown in Figure 3-23 expressed HLA-B*0702 and therefore the low or absent responses restricted by HLA-A*0201 cannot be explained by the dominance of HLA-B*0702 restricted responses in individuals expressing HLA-A*0201 and HLA-B*0702 that was reported previously (Lacey *et al.*, 2003). However, as outlined above, all three patients are likely to be protected by yet another response than that targeting HLA-A2/pp65. Conclusions on a possible hierarchy of immunodominance between the different CMV specific $CD8^+$ T cells that can be drawn from these results will be discussed in section 3-3.

HLA-A2/pp65 specific $CD8^+$ T cell responses in two patients who did not recover critical levels of total $CD4^+$ T helper cell counts are shown in Figure 3-24. The importance of $CD4^+$ T helper cell counts for the functionality of antiviral $CD8^+$ T cells is explained in detail in section 3-3, from page 238. Low numbers of $CD4^+$ T helper cells are likely to influence protection from CMV irrespective of CMV specific $CD8^+$ T cell levels in these patients.

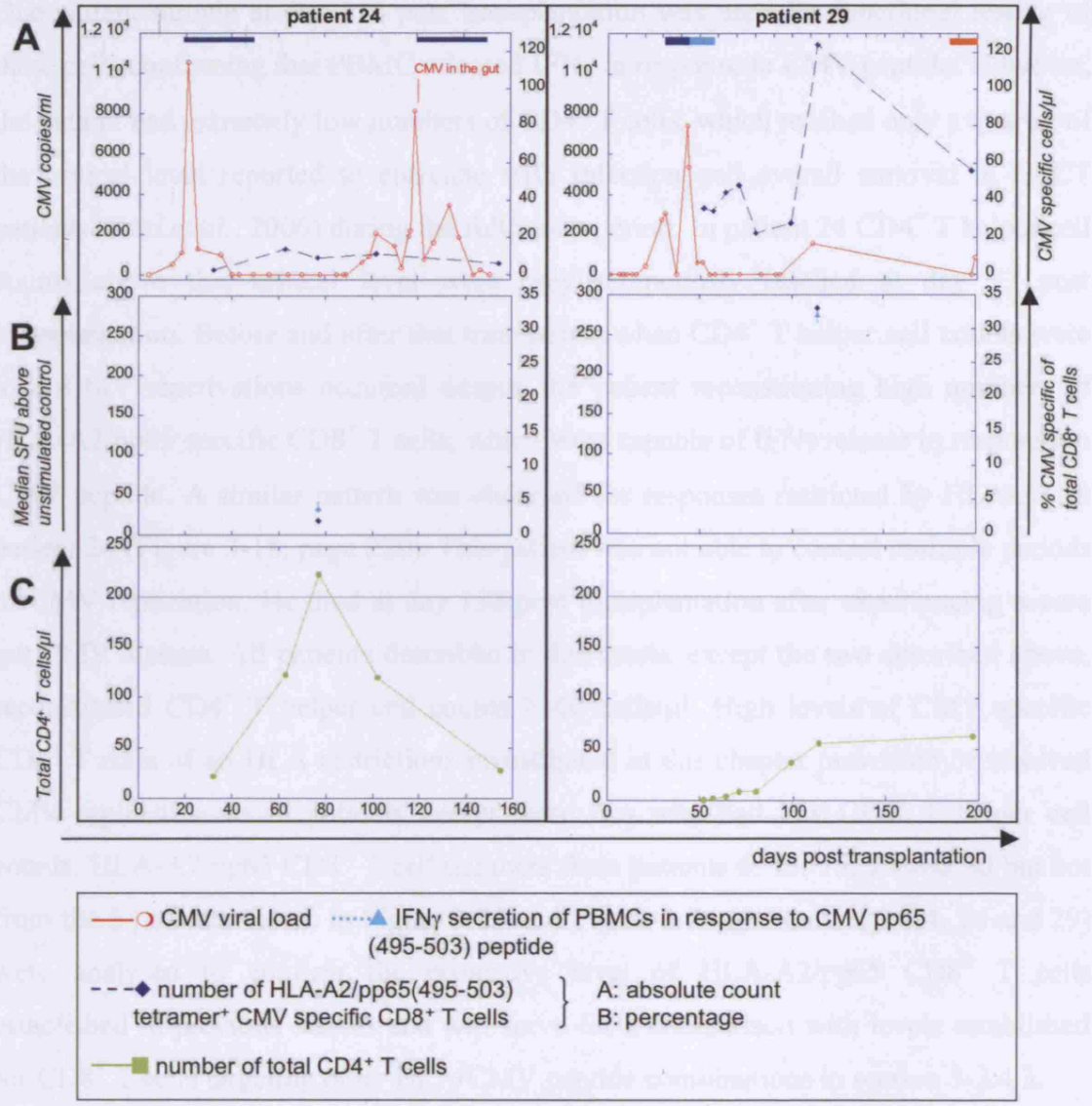


Figure 3-24 Monitoring of HLA-A2/pp65 specific CD8$^+$ T cell responses and lack of CD4$^+$ T cell help in patients 24 and 29

HLA-A2/pp65 specific CD8$^+$ T cell responses are shown in a similar way as described for Figure 3-20 on page 227. ● CD4$^+$ T cell counts are shown in graph C. The dotted line represents the critical value of CD4$^+$ T cells shown to correlate with infection and overall survival in HSCT patients (Kim *et al.*, 2006). Horizontal yellow-red and blue bars indicate immunosuppressive (■ Prednisolone) and antiviral (■ Ganciclovir, ■ Foscarnet) treatment respectively.

Patient 29 had very high levels of HLA-A2/pp65 specific CD8$^+$ T cells (note higher scale in Figure 3-24 than in previous figures) despite continuous CMV replication. HLA-A2/pp65 specific CD8$^+$ T cells made up an unusually high frequency of 69.4 % of the total CD8$^+$ T cell population at day 64 post transplantation. This translates into 44.4 cells/µl of peripheral blood at that time. The frequency of those cells subsequently decreased to 25.5 % of total CD8$^+$ T cells, which translates into 56.1 cells/µl due to an increased number of total CD8$^+$ T cells in the patient at day 197 post transplantation.

One patient sample at day 114 post transplantation was used for functional testing of these cells confirming that PBMC released IFNγ in response to CMV peptide. However, the patient had extremely low numbers of $CD4^+$ T cells, which reached only a quarter of the critical level reported to correlate with infection and overall survival in HSCT patients (Kim *et al.*, 2006) during the follow-up period. In patient 24 $CD4^+$ T helper cell counts above this critical level were only temporarily reached at day 77 post transplantation. Before and after that time point (when $CD4^+$ T helper cell counts were low) CMV reactivations occurred despite the patient reconstituting high numbers of HLA-A2/pp65 specific $CD8^+$ T cells, which were capable of IFNγ release in response to CMV peptide. A similar pattern was observed for responses restricted by HLA-A1 in patient 24 (Figure 3-18, page 220). This patient was not able to control multiple periods of CMV replication. He died at day 158 post transplantation after experiencing severe gut CMV disease. All patients described in this thesis, except the two described above, reconstituted $CD4^+$ T helper cell counts ≥160 cells/µl. High levels of CMV specific $CD8^+$ T cells of all HLA restrictions investigated in this chapter prevented or resolved CMV replication in all patients except these two who had low $CD4^+$ T helper cell counts. HLA-A2/pp65 $CD8^+$ T cell numbers from patients 6, 13, 15, 23 and 30 but not from the 5 patients shown in Figure 3-23 and Figure 3-24 (patients 1, 2, 24, 26 and 29) were analysed to confirm the protective level of HLA-A2/pp65 $CD8^+$ T cells established in previous studies and will serve for a comparison with levels established for $CD8^+$ T cells targeting other HLA/CMV peptide combinations in section 3-2.4.3.

3-2.4.3 Statistical analysis of the protective level of HLA-A2 restricted CMV specific $CD8^+$ T cells and summary

HLA-A2/pp65 specific $CD8^+$ T cell responses were studied within this project to allow for a comparison of responses towards new targets described in the previous sections with this archetype. Cell numbers observed in patients 6, 13, 15, 23 and 30 (compare section 3-2.4.2: Figure 3-20 on page 227, Figure 3-21 on page 229 and Figure 3-22 on page 230) are statistically analysed for their correlation with CMV viral load in patients here with results shown in Figure 3-25.

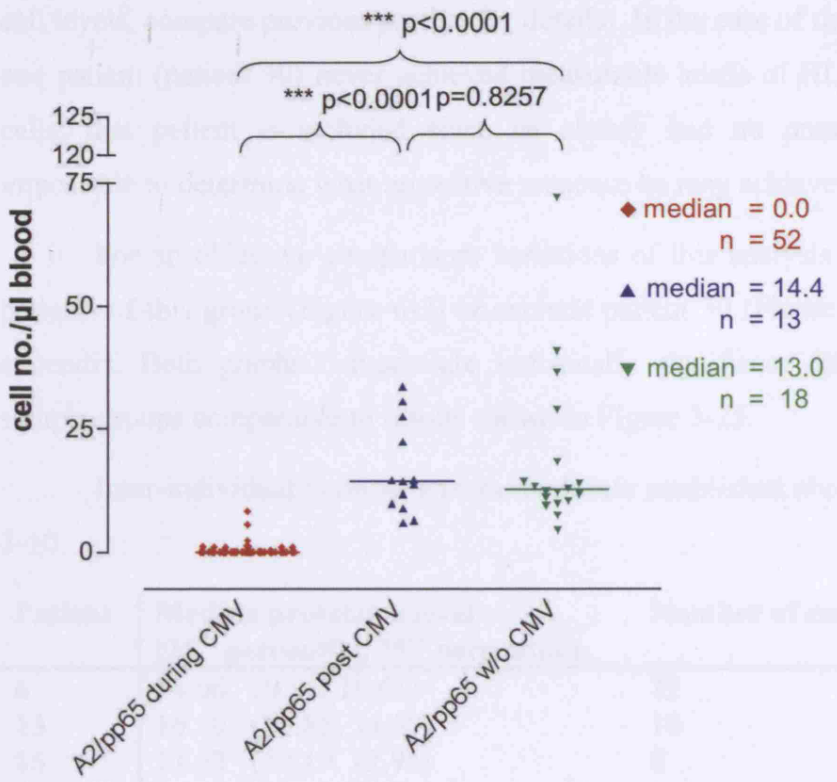


Figure 3-25 Statistical analysis of HLA-A2/pp65 specific CD8$^+$ T cell responses

This figure illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in patients 6, 23 and 30 measured post transplantation until the last observed CMV reactivation (◆ during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in those patients that resolved their CMV reactivations (patients 6 and 23) (▲ post CMV) in comparison to absolute numbers of tetramer binding cells in patients 13 and 15 who did not reactivate CMV (▼ w/o CMV). Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

Figure 3-25 demonstrates that HLA-A2/pp65 specific CD8$^+$ T cell numbers broadly correlated inversely with the viral load in HSCT patients. Most T cell responses during the period of CMV replication in patients 6, 23 and 30 were not detectable, compared to significantly higher levels ($p<0.0001$) measured after the period of CMV reactivation (median 14.4 cells/µl). These responses were similar to those measured in patients 13 and 15, who did not reactivate the virus (median 13.0 cells/µl).

One limitation of this analysis is that the "post CMV" and "w/o CMV" groups contain only responses from those patients in whom HLA-A2/pp65 specific T cell levels were measurable; other patients were considered to be protected by other responses (patients 1 and 2 demonstrated HLA-B35/pp65 specific CD8$^+$ T cells, and patient 26 reconstituted reasonable numbers of total CD4$^+$ and CD8$^+$ T cells) or to be not informative due to other reasons (patients 24 and 29 did not recover critical T helper

cell levels, compare previous section for details). In the case of the "reactivating" group, one patient (patient 30) never achieved measurable levels of HLA-A2/pp65 specific T cells; that patient is included since he clearly had no protective response. It is impossible to determine what protective response he may achieve in the future, if any.

For an objective comparison, variations of this analysis that either include all patients of this group (Figure 6-7) or exclude patient 30 (Figure 6-8) are shown in the appendix. Both graphs demonstrate statistically significant differences between the sample groups comparable to results shown in Figure 3-25.

Inter-individual variations from the levels established above are shown in Table 3-10.

Patient	Median protective level (25^{th} percentile, 75^{th} percentile)	Number of samples
6	14.06 (9.55, 16.61)	12
13	16.10 (11.85, 34.60)	10
15	11.53 (10.19, 12.96)	8
23	*33.63* -	*1*

Table 3-10 Protective levels of HLA-A2/pp65 specific $CD8^+$ T cell responses in individual patients

This table lists the median values of absolute numbers of tetramer binding cells/µl of peripheral blood in patients 6, 23 and 30 measured from the peak of the last CMV reactivation onwards and for absolute numbers of tetramer binding cells in patient 13 and 15 who did not reactivate CMV (in italics: calculated from <3 samples) along with the number of samples used to calculate each median value.

Table 3-10 demonstrates a slightly higher level of cells correlating with protection against CMV for patient 23 than others. The level for patient 23 was calculated from a single sample value. That sample was the first sample to be obtained after a CMV replication peak and therefore likely represented the peak of the T cell response (Figure 3-21) whereas calculations for other patients incorporated peak values and values obtained from samples taken after T cell responses had diminished to a lower sustained value.

It can be concluded that the level of HLA-A2/pp65 specific $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation in patients from this study ($13x10^6$/L, compare Figure 3-26 on page 241) is similar to protective levels of $10x10^6$ (Cwynarski *et al.*, 2001) to $20x10^6$ (Aubert *et al.*, 2001) cells/L of blood

established for HLA-A*0201 restricted $CD8^+$ T cell responses to CMV pp65 (495-503) in HSCT patients previously.

The protective level of HLA-A2/pp65 specific $CD8^+$ T cell responses in HSCT patients established above was compared to the protective levels of CMV specific $CD8^+$ T cell responses restricted by HLA-B35, HLA-A24 and HLA-A1 that were established in the previous sections and a statistical analysis is shown in Figure 3-26 in section 3-3 on page 241.

Similar to the CMV specific $CD8^+$ T cell responses restricted by other HLA types described in the previous sections, HLA-A2/pp65 specific $CD8^+$ T cell response levels were also analysed in six healthy CMV seropositive volunteers (Table 3-11).

Healthy volunteer	HLA-A2/pp65 specific cells as a percentage of $CD8^+$ T cells	HLA-A2/pp65 specific cells per microlitre of blood
1	0.81	2.59
5	0.61	2.96
6	0.42	1.35
8	3.26	22.14
10	1.28	7.42
15	0.23	1.18

Table 3-11 HLA-A2/pp65 specific $CD8^+$ T cell levels in healthy volunteers

This table lists identification numbers of healthy volunteers and the HLA-A2/pp65 specific $CD8^+$ T cell counts detected in peripheral blood of these volunteers by use of tetramer staining. Counts are represented as a percentage of the total $CD8^+$ T cell population and as a number per volume of blood.

Table 3-11 demonstrates the frequency of HLA-A2/pp65 specific $CD8^+$ T cells observed in healthy control samples. This translates to a mean frequency of 1.1 % of HLA-A2/pp65 specific $CD8^+$ T cells (median 0.71 %, range 0.23 - 3.26 %) and mean absolute number of 6.27×10^6 HLA-A2/pp65 specific $CD8^+$ T cells/L (median 2.78×10^6 T cells/L, range 1.18 - 22.14) was observed in healthy control samples (n = 6).

The level of HLA-A2/pp65 specific $CD8^+$ T cell responses that was observed in healthy volunteers during this study is similar to findings published previously. Aubert and colleagues reported a mean frequency of 1.3 % of HLA-A2/pp65 specific $CD8^+$ T cells (median 0.935 %, n = 13, range 0.29 - 5 %) and an absolute number estimated at a mean of 8.7×10^6 HLA-A2/pp65 specific $CD8^+$ T cells/L. This value was based on an average $CD8^+$ T cell count of 0.67 (range 0.2 - 1.14) $\times 10^9$ T cells/L within that study (Aubert *et al.*, 2001).

HLA-A2/pp65 specific $CD8^+$ T cell numbers measured in healthy individuals during this study are lower than the level observed in HSCT patients ($p = 0.0066$), compare Figure 3-26 on page 241.

3-3 Discussion

The main aim of this chapter was to evaluate and compare *ex vivo* CMV specific $CD8^+$ T cells with different HLA/peptide targets in patients post HSCT.

3-3.1 Correlation of CMV specific $CD8^+$ T cells with protection from CMV in HSCT patients

Overall a broad inverse correlation was observed between the numbers of tetramer binding $CD8^+$ T cells and the CMV viral load at the time of sampling in patients, who had reconstituted at least 160 $CD4^+$ T helper cells/µl blood. This suggests functionality of CMV specific $CD8^+$ T cells *in vivo*. Although limited cell numbers did not allow for assessment of cytotoxicity, IFNγ ELISpot analysis demonstrated cytokine release of PBMC in response to the same CMV peptides that were used to generate tetramers. This is important for the protective capacity of these cells in patients since it has been suggested that a fraction of CMV specific $CD8^+$ T cells may be non-functional (Engstrand *et al.*, 2003, Ouyang *et al.*, 2003, Zhang *et al.*, 2003).

Numbers of $CD8^+$ T cells targeting a specific HLA/CMV peptide combination were analysed separately in different sections throughout this chapter. Each HLA specific section includes a statistical analysis of T cell responses grouped according to the viral load observed at the time of sampling in patients. Not all of the responses measured in HSCT patients could be used for a meaningful statistical analysis. Four exceptions were considered which are explained below.

Firstly, some responses were observed at very low frequency or in a very limited number of patients not allowing for quantitative statistical analysis. Under HLA-A*0101 restriction, frequencies of $CD8^+$ T cells targeting CMV pp65 (341-349) were insignificant in all patients, and $CD8^+$ T cells targeting CMV pp65 (363-373) were absent or of lower frequency than $CD8^+$ T cells targeting CMV pp50 (245-253). Due to observation of significant numbers of CMV pp65 (363-373) specific $CD8^+$ T cells that may have conferred protection against CMV in only two patients, a statistical analysis was only possible for HLA-A1/pp50 but not HLA-A1/pp65 specific $CD8^+$ T cells

(compare section 3-2.3.3, page 221). Under HLA-A*2402 restriction, frequencies of $CD8^+$ T cells targeting CMV pp65 (113-121) were insignificant in all patients.

Secondly, responses in CMV seronegative patients who received a transplant from a CMV seropositive donor and did not experience a primary infection were omitted from statistical analysis.

Thirdly, patients in whom CMV was controlled despite a lack of detection of significant CMV specific $CD8^+$ T cells with the relevant tetramer were observed in this and other (Aubert *et al.*, 2001) studies. Findings from HLA-A2 expressing CMV seropositive populations reported HLA-A2/pp65 specific $CD8^+$ T cells in 75 % (Gratama *et al.*, 2001) to 77 % (Komatsu *et al.*, 2003) of individuals tested previously. Provided that total $CD8^+$ T cell counts were reconstituted, $CD8^+$ T cells specific for other HLA/CMV targets were likely to protect such patients. This was shown for some patients in this chapter, in whom staining with one of the other reagents of the tetramer panel was positive. CMV specific immune cells not covered by the tetramers used in this study likely protected others. Responses measured in those patients were not informative (undetectable responses cannot be used to establish response levels that may be protective in patients) and were therefore omitted from subsequent statistical analysis to establish protective levels of CMV responses.

Fourthly, CMV specific $CD8^+$ T cell responses were not statistically analysed in patients with extremely low $CD4^+$ T cell levels. Although $CD8^+$ T cells can resolve acute virus infections without $CD4^+$ T cell help, long-term control of persistent infection requires $CD4^+$ T cells (Matloubian *et al.*, 1994). There are a number of mechanisms by which $CD4^+$ T cells can influence the $CD8^+$ T cell responses:

- Findings from Janssen and colleagues suggested that the presence of $CD4^+$ T cells during priming is dispensable for primary expansion and differentiation of $CD8^+$ T cells but not for secondary $CD8^+$ T cell expansion upon re-encounter with antigen (Janssen *et al.*, 2003).
- CMV specific $CD8^+$ T cells are dependent on $CD4^+$ T cells that provide essential IL2 (Salkowitz *et al.*, 2004). This is especially pronounced for highly differentiated $CD8^+$ T cells that have lost CD28 expression (Topp *et al.*, 2003). $CD28^-$ $CD8^+$ T cells are increased in the CMV specific $CD8^+$ memory T cell pool (Gillespie *et al.*, 2000). CD28 interacts with CD80 and CD86 on APC and co-engagement of CD28 and TCR results in increased sensitivity to TCR

stimulation due to increased stability of the immunological synapse (Viola and Lanzavecchia, 1996) and survival of T cells after stimulation (due to increased expression of anti-apoptotic proteins and IL2) (Boise *et al.*, 1995).

- It was reported that a lack of $CD4^+$ T cells could result in silencing of persistent antiviral $CD8^+$ T cells, which proliferate but are unable to kill the virus (Zajac *et al.*, 1998).
- Besides providing IL2 as a growth and survival factor (Vella *et al.*, 1998), $CD4^+$ T cells are important for conditioning APC through CD40-CD40L interactions (Schoenberger *et al.*, 1998). Therefore it is likely that in the absence of sufficient helper activity (and contact of T helper cells with dendritic cells through CD40-CD40L interactions) dendritic cells may not provide sufficient costimulatory signals to $CD8^+$ T cells, thus disrupting their normal function (Kalams and Walker, 1998).
- $CD8^+$ T cells generally recognise endogenously processed antigen presented via MHC class I. However, exogenous antigen may also enter the otherwise endogenous class I presentation pathway, a process know as cross presentation (Bevan, 1976). CMV cross presentation has been demonstrated by cell entry of defective envelope particles called dense bodies (Pepperl *et al.*, 2000). In turn, induction of $CD8^+$ T cells by cross-priming has also demonstrated to require $CD4^+$ T cell help, a process which has been termed conditioning (Ridge *et al.*, 1998) or licensing (Lanzavecchia, 1998) of DC.

Thus it is clear that memory $CD8^+$ T cells may be compromised in disease states that result in deficits in $CD4^+$ T cell numbers or function. $CD8^+$ T cells have a more rapid recovery rate than $CD4^+$ T cells leading to an inversion of the normal CD4/CD8 ratio (2:1) early post transplantation (Dumont-Girard *et al.*, 1998, Singer *et al.*, 1983). Patients lacking critical numbers of $CD4^+$ T cells post HSCT may not be able to translate the number of CMV specific $CD8^+$ T cells into protection against CMV. Absolute $CD4^+$ and $CD8^+$ T cell counts were measured by TruCOUNT analysis or were retrieved from routine hospital counts in a minority of patients. Patients 24 and 29 did not recover $CD4^+$ T helper cell counts of $\geq$160 cells/μl like other patients in this study. This cut-off level is similar to the threshold of 200 $CD4^+$ T cells/μl blood that was reported to correlate with infection and overall survival in HSCT patients (Kim *et al.*, 2006). CMV specific $CD8^+$ T cell responses from patients 24 and 29 were therefore

omitted from statistical analysis aiming at establishing CMV specific $CD8^+$ T cells correlating with protection in patients.

3-3.2 CMV specific $CD8^+$ T cells correlate with protection from CMV in HSCT patients at different levels depending on the HLA/peptide combination but may not mediate protection

The protective levels established in the HLA specific analysis of this chapter vary for different HLA/peptide combinations. This is illustrated in Figure 3-26, which summarises data shown in the previous HLA specific sections.

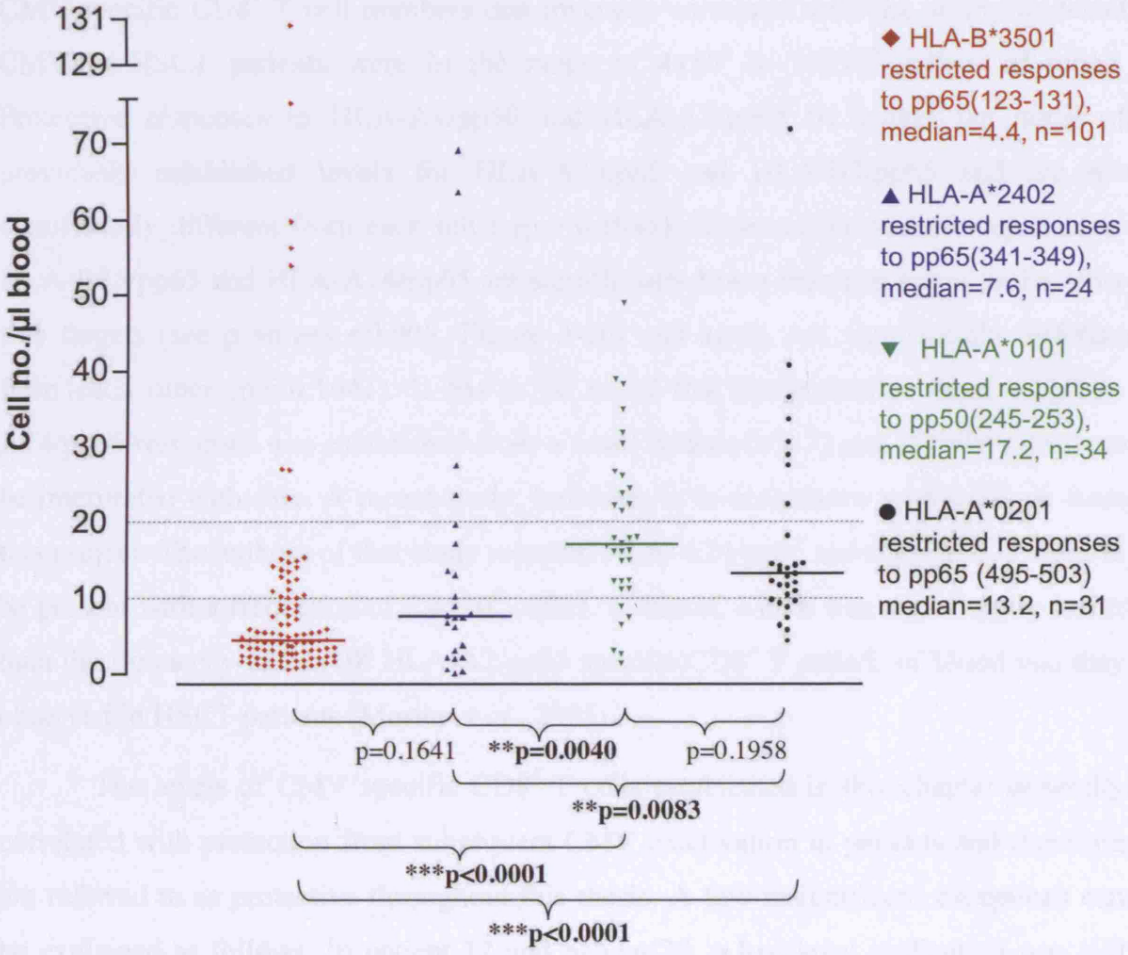


Figure 3-26 Comparison of protective levels of CMV specific $CD8^+$ T cell responses with different HLA/peptide targets in HSCT patients

This represents an illustration of the absolute numbers of tetramer binding cells/µl of peripheral blood measured post transplantation since the last observed CMV reactivation or during the entire follow-up period in patients who did or did not reactivate CMV respectively. Responses were grouped into columns according to their HLA/peptide target specificity with the median and number of responses analysed shown in the legend on the right. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

Since HLA-specific analysis in this chapter demonstrated similar levels of responses in patients who had resolved CMV reactivation and patients who did not reactivate, these groups have been combined in Figure 3-26. Protective levels established previously (Cwynarski *et al.*, 2001, Aubert *et al.*, 2001) for HLA-A*0201 and/or HLA-B*0702 restricted CMV pp65 specific $CD8^+$ T cell responses are indicated as a dashed line in Figure 3-26. Samples were analysed from patients with various diseases and transplant modalities (compare Table 2-1 on page 99) during this project. Nevertheless the statistical analysis was powerful enough to detect significant differences between $CD8^+$ T cell responses targeting different HLA/CMV peptide combinations. The medians of CMV specific $CD8^+$ T cell numbers that inversely correlated with the ability to detect CMV in HSCT patients were in the range of $4x10^6$ to $17x10^6$ cells/L of blood. Protective responses to HLA-A1/pp50 and HLA-A2/pp65 lie within the range of previously established levels for HLA-A2/pp65 and HLA-B7/pp65 and are not significantly different from each other ($p = 0.1985$). However, protective responses to HLA-B35/pp65 and HLA-A24/pp65 are significantly lower than responses to the other two targets (see p values <0.008, Figure 3-26) and again, not significantly different from each other ($p = 0.1641$). It has to be noted that the protective level of HLA-A24/pp65 responses was established from a small dataset ($n = 3$) and therefore needs to be interpreted with care. A recent study, however, is in accordance with findings from this project. The authors of that study reported HLA-A24/pp65 specific $CD8^+$ T cells to be present with a frequency of $0.4x10^6$ cells/L of blood, which was significantly lower than the frequency of $23x10^6$ HLA-A2/pp65 specific $CD8^+$ T cells/L of blood that they observed in HSCT patients (Morita *et al.*, 2005).

The levels of CMV specific $CD8^+$ T cells established in this chapter generally correlated with protection from subsequent CMV reactivation in patients and therefore are referred to as protective throughout this thesis. A few insignificant exceptions can be explained as follows. In patient 12 and patient 22, a low-level replication was still observed shortly after protective levels of CMV specific $CD8^+$ T cells had been reached. However, these were resolved quickly and may simply reflect the time needed by $CD8^+$ T cells to effectively impact on CMV replication. In patient 12 it is possible that a temporary drop of the response below the protective level has been missed between samples taken at day 59 and 80 post transplantation because CMV specific $CD8^+$ T cell numbers decreased until day 59 and increased from day 80 (Figure 3-7, page 198). Alternatively the low-level PCR result of 931 and 236 CMV copies/ml blood at day 73

and 77 post HSCT in patient 12 respectively can be considered insignificant (which is consistent with antiviral treatment guidelines described in section 2-2.5). In patient 22 the functionality of responses that were measured may have been partially compromised due to Prednisolone treatment at the time of observed low-level CMV PCR results (202, 406 and 828 copies/ml at day 56, 87 and 108 post HSCT, which again can be considered insignificant) because further CMV replication was prevented after cessation of this immunosuppressive treatment (Figure 3-15, page 215). This project did not aim to prove the protective capacity of CMV specific $CD8^+$ T cells in HSCT patients *per se*. Rather this study was performed based on previous findings that reported evidence for CMV specific $CD8^+$ T cells being protective (Reusser *et al.*, 1991, Walter *et al.*, 1995) and aimed to compare the numbers of different CMV specific $CD8^+$ T cells (targeting different HLA/peptide combinations) that inversely correlate with the ability to detect CMV reactivation in patients. The results of investigations described in this chapter do not prove that CMV specific $CD8^+$ T cells mediated protection. The magnitude of $CD8^+$ T cells targeting a specific CMV epitope is inversely correlated with viral load and therefore correlates with protection in patients, but it may be possible that protection was mediated by other immune responses either individually or in combination with the cell populations detected. These could be cells with higher sensitivity, which may expand and/or function at a lower viral load than the CMV specific $CD8^+$ T cells measured using the panel of tetramers available for this study. If so the CMV specific $CD8^+$ T cells measured would be a surrogate marker. Due to their inverse correlation with viral load, however, cell levels measured are useful to indicate if the immune status in patients is sufficient for the protection from CMV reactivation. Therefore in patients with at least 160 $CD4^+$ T helper cells/µl blood present, CMV specific $CD8^+$ T cell levels measured are a marker of protection independently of their capacity to mediate protection.

A possibility to prove that $CD8^+$ T cells targeting a specific CMV epitope are mediating protection from CMV *in vivo* is to adoptively transfer those cells to patients with uncontrolled CMV reactivations and establish whether the transfer may result in subsequent protection from any further CMV reactivations. Findings from patients 24 and 29 in this study suggest that efficiency of CMV specific $CD8^+$ T cells would require the presence of $CD4^+$ T cells. Cobbold and colleagues demonstrated that CMV reactivation resolved completely after adoptive transfer of various CMV specific $CD8^+$ T cells in eight patients and markedly reduced CMV viral load in a single patient after

adoptive transfer of HLA-B35/pp65 specific $CD8^+$ T cells (Cobbold *et al.*, 2005). This merits further adoptive transfer studies with each subset of CMV specific $CD8^+$ T cells in a larger patient cohort to confirm the protective capacity of the different subsets of CMV specific $CD8^+$ T cells.

3-3.3 Correlation of CMV specific $CD8^+$ T cells with protection at different levels depending on the HLA/peptide combination can also be observed in healthy virus carriers

As Riddell and Greenberg reviewed, healthy CMV seropositive individuals maintain high levels of CMV specific T cells which they suggest may be required to control intermittent episodes of virus reactivation (Riddell and Greenberg, 2000), the occurrence of which was subsequently confirmed (Ling *et al.*, 2003). During this project CMV specific $CD8^+$ T cells were measured in a small number of CMV seropositive healthy volunteers expressing the HLA tissue types under investigation. The results from this analysis demonstrate (similar to observations in HSCT patients) different cells levels depending on the antigen specificity of cells. Figure 3-27 demonstrates significant differences between CMV specific $CD8^+$ T cell responses targeting different HLA/CMV peptide combinations.

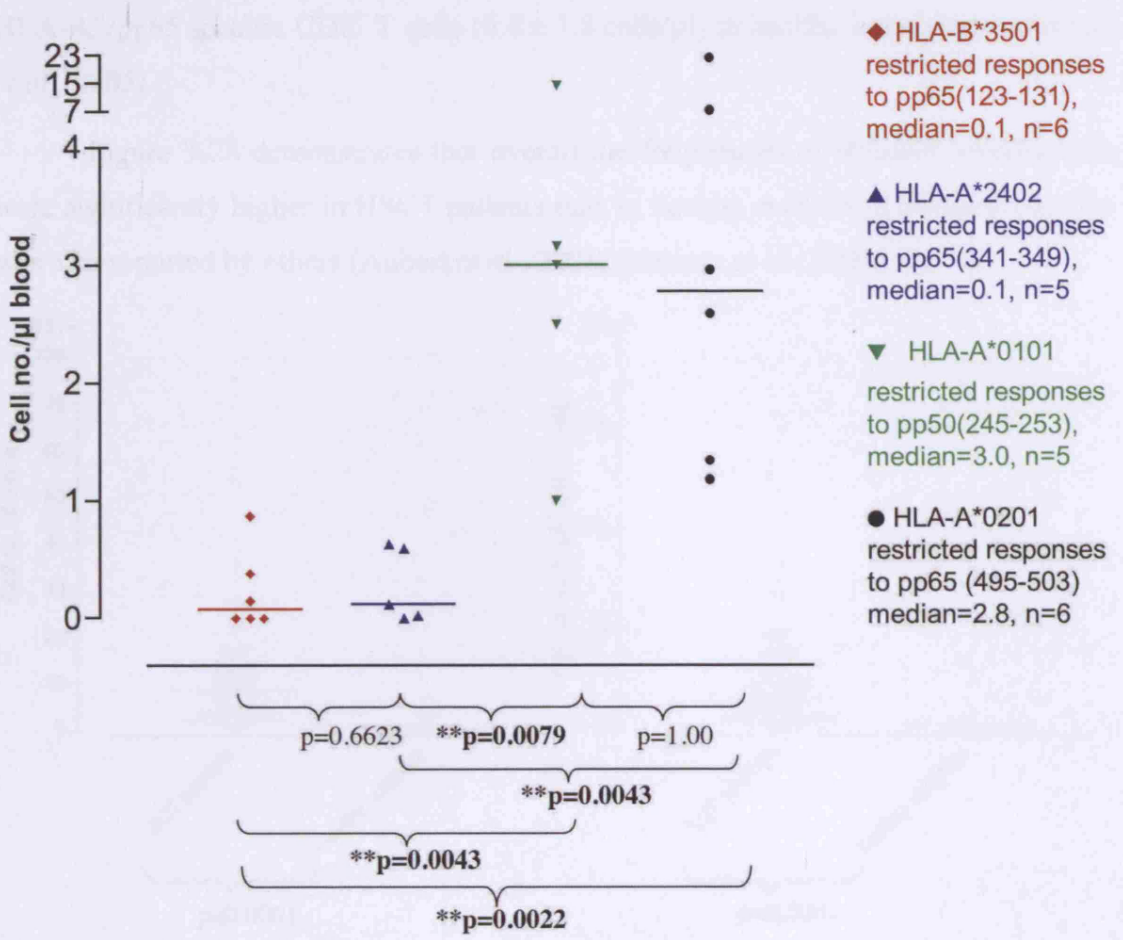


Figure 3-27 Comparison of CMV specific CD8$^+$ T cell counts targeting different HLA/peptide combinations in healthy individuals

This figure represents CMV specific responses observed in healthy individuals. It illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood measured in CMV seropositive individuals expressing the relevant HLA tissue types. Responses were grouped into columns according to their HLA/peptide target specificity with the median and number of responses analysed shown in the legend on the right. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

Similar to HSCT patients (Figure 3-26), frequencies of CMV specific CD8$^+$ T cells targeting HLA-B35/pp65 or HLA-A24/pp65 are significantly lower (median 0.08 cells/µl blood for both) than those targeting HLA-A1/pp50 or HLA-A2/pp65 (median 3.00 and 2.78 cells/µl blood respectively) in healthy volunteers (Figure 3-27, $p<0.02$). Frequencies of two high-responders and three low-responders amongst the healthy virus carriers (compare Table 3-9, page 224) result in a median of 0.41 HLA-A1/pp65 specific CD8$^+$ T cells/µl blood. These results are in accordance with findings in a recent publication describing lower frequencies of HLA-B35/pp65, HLA-A24/pp65 or HLA-A1/pp65 specific CD8$^+$ T cells (0.9 ± 0.6, 0.1 ± 0.1 and 1.3 ± 0.5 cells/µl) than

HLA-A2/pp65 specific $CD8^+$ T cells (6.4 ± 1.8 cells/µl) in healthy individuals (Lidehall *et al.*, 2005).

Figure 3-28 demonstrates that overall the frequencies of tetramer binding cells were significantly higher in HSCT patients than in healthy controls, a phenomenon that was also reported by others (Aubert *et al.*, 2001, Gratama *et al.*, 2001).

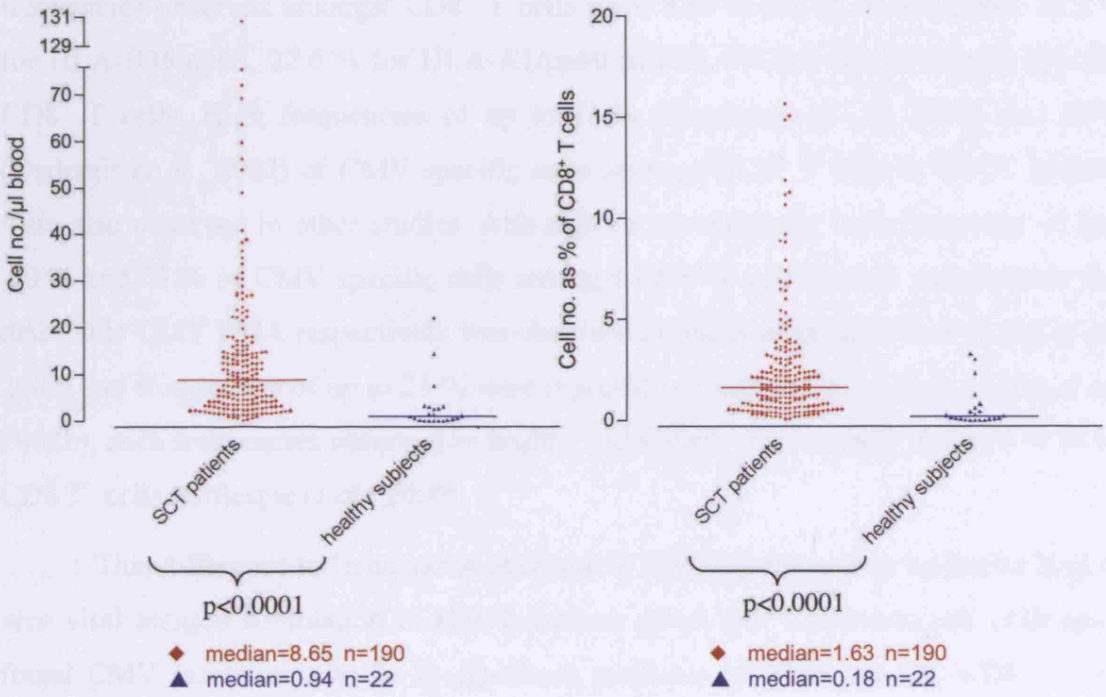


Figure 3-28 Comparison of CMV specific $CD8^+$ T cell counts in protected HSCT patients and healthy individuals

The numbers of tetramer binding cells/µl (left graph) and the numbers of tetramer binding cells as a percentage of $CD8^+$ T cells (right graph) measured in CMV seropositive individuals are illustrated. Responses were separated into measurements in those HSCT patients that were regarded as protected ("SCT patients", pooled data from Figure 3-26) and measurements in healthy virus carriers ("healthy subjects", pooled data from Figure 3-27) with the median and number of responses analysed shown below the graphs. A statistically comparison was performed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

Chalandon and colleagues were using tetramers with HLA-A*0101/CMV pp65 (363-373), HLA-A*0201/CMV pp65 (405-503) and HLA-B*0702/CMV pp65 (265-275 and 417-426) specificities and observed an average of 6.7 ± 4.6 % tetramer binding cells amongst total $CD8^+$ T cells in HSCT patients in contrast to an average of 1.3 ± 1.6 % in patients before transplantation and an average of 0.7 ± 0.7 % in CMV seropositive donors (Chalandon *et al.*, 2006). Data shown in Figure 3-28 translate into an average of 2.4 ± 2.8 % tetramer binding cells amongst total $CD8^+$ T cells in HSCT patients in contrast to an average of 0.6 ± 1.0 % in healthy CMV carriers. The average of tetramer

binding cells in HSCT patients observed in this study is lower than in the study by Chalandon and colleagues, which likely results from the use of tetramers with specificity for cells present at lower levels (HLA-B35/pp65 n = 101, total n = 190).

In this study supranormal frequencies of CMV specific $CD8^+$ T cells were observed at the peak of responses after CMV reactivation in HSCT patients. Maximum frequencies observed amongst $CD8^+$ T cells were 8.89 % for HLA-A24/pp65, 20.2 % for HLA-B35/pp65, 22.6 % for HLA-A1/pp50 and 69.4 % for HLA-A2/pp65 specific $CD8^+$ T cells. High frequencies of up to 21 % (Cwynarski *et al.*, 2001) and 40 % (Ozdemir *et al.*, 2002) of CMV specific cells amongst $CD8^+$ T cells in HSCT patients were also observed in other studies. Although an exceptionally high frequency of 26 - 50 % and 65 % of CMV specific cells amongst $CD8^+$ T cells during undetectable and detectable CMV DNA respectively was observed in one healthy individual (Lang *et al.*, 2002) and frequencies of up to 23 % were reported in some old individuals (Khan *et al.*, 2002b), such frequencies observed in healthy individuals were usually below 5 % of all CD8 T^+ cells (Gillespie *et al.*, 2000).

This difference to frequencies observed in HSCT patients may be due to high *in vivo* viral antigen stimulation in HSCT patients given that Cwynarski and colleagues found CMV reactivation to be a significant predictor of CMV specific $CD8^+$ T cell reconstitution in univariate analysis (Cwynarski *et al.*, 2001). Findings described here, however, demonstrate similar levels of CMV specific $CD8^+$ T cells in non-reactivating patients and patients post CMV reactivation, which are higher than levels observed in healthy virus carriers. The finding of similar levels of CMV specific $CD8^+$ T cells in non-reactivating HSCT patients and patients post CMV reactivation is consistent with reports by others (Gallez-Hawkins *et al.*, 2005). This suggests a transplant related rather than stimulation related reason behind the higher level of viral $CD8^+$ T cells in HSCT patients than in healthy controls. T cell homeostasis is regulated by the competition of different T cells for endogenous stimuli like cytokines and access to APC (Kedl *et al.*, 2000). T cells may compete when the number of APC or antigen is limited and the T cells are present in numbers high enough to influence one another. This competition can occur between $CD8^+$ T cells responding to the same HLA/antigen (direct competition) or, more rarely, between $CD8^+$ T cells responding to different HLA/antigen (cross competition) (Kedl *et al.*, 2003). This is discussed in more detail in section 1-11.2. Upon transfer of cells to a lymphopenic environment such as conditioned HSCT recipients, T cells proliferate until the cellularity of the T cell compartment has been

restored (Ernst *et al.*, 1999, Goldrath *et al.*, 2000). The homeostatic drive in addition to CMV antigen stimulus in HSCT patients may explain the high levels of CMV specific $CD8^+$ T cells observed. After resolved CMV replication episodes, T cells may not contract to the same levels observed in healthy virus carriers but remain at higher levels in HSCT patients due to higher availability of space and endogenous stimuli. This leads to an inflation of CMV specific $CD8^+$ T cells over time, which is discussed further in Chapter 4.

3-3.4 Potential origin and crossreactivity of CMV specific $CD8^+$ T cells in recipients of HSCT

Recipients of allogeneic stem cell transplantation can reconstitute CMV specific $CD8^+$ T cells from different cell populations. Following receipt of unmanipulated stem cells most T cells are of donor origin (Roux *et al.*, 1996, Gandhi *et al.*, 2003a). They may stem from mature donor T cells transfused with the graft or from donor haematopoietic progenitors in the graft that underwent TCR gene rearrangement in the thymus of the recipient and gave rise to naive T cells. Alternatively CMV specific $CD8^+$ T cells can originate from mature recipient T cells that survived the conditioning regimen (Butturini *et al.*, 1986) and escaped attack by donor T cells if the graft was thoroughly depleted of those cells (Roux *et al.*, 1992). This may be more pronounced in HSCT using reduced-intensity conditioning.

Only 17 % of CMV seronegative recipients who received transplants from seropositive donors were reported to experience CMV infection (Goodrich *et al.*, 1994). Consistent with that infection was not observed in all three patients fulfilling these criteria in this study.

Delayed reconstitution of CMV specific $CD8^+$ T cells was reported post HSCT from CMV seronegative donors (Cwynarski *et al.*, 2001, Gandhi *et al.*, 2003a). In accordance with that CMV specific $CD8^+$ T cell reconstitution was absent or delayed in most CMV seropositive patients who received transplants from seronegative donors in this study. Two patients, patient 7 and 15, however, had 10 % and 3 % CMV specific cells detected within their $CD8^+$ T cell populations as early as 46 and 22 days post transplantation from a CMV seronegative donor respectively. To my knowledge this is the first observation of early detection of such cells in CMV seropositive recipients who did not experience CMV replication after receiving transplants from seronegative donors. $CD8^+$ thymic emigrants can be observed by 2 months after allogeneic HSCT

(Weinberg *et al.*, 2001), but it has been suggested that the contribution of newly generated T cells from the naïve T cell pool may be negligible during the first 6 months for $CD4^+$ T cells and during the first 9 months post transplantation for $CD8^+$ T cells (Fallen *et al.*, 2003). Therefore it is likely that CMV specific $CD8^+$ T cells in patient 7 and 15 observed during this project derived from mature cells rather than from donor haematopoietic progenitors. The graft received by myeloablated patient 7 was not T cell depleted suggesting that cells may have been of donor rather than recipient mature T cell origin. In comparison, patient 15 received a non-myeloablative transplant involving reduced-intensity conditioning, which was reported to induce mixed T cell chimaerism by 4 weeks post HSCT in a majority of patients (Laport *et al.*, 2008). Exposure to antigen apparently was not required for maintenance of tetramer binding T cells because CMV DNAemia was not detected in peripheral blood in either patient. This may not preclude subclinical CMV reactivation at localised tissue sites. CMV specific $CD8^+$ T cells demonstrating dim staining with tetramer coincided with the occurrence of acute GvHD in patient 7 (Figure 3-1, page 180 and Figure 3-2, page 184). It is therefore suspected that T cell receptors of those cells may have been crossreactive with another antigen in the recipient. The possibility of virus specific $CD8^+$ T cells cross-reacting with alloantigen was reported previously (Gamadia *et al.*, 2004, Nahill and Welsh, 1993). Alloreactive cells may directly participate in GvHD or the cytokines released in response to GvHD may contribute to the maintenance of CMV specific T cells. Allo-crossreactivity has been suggested previously for CMV specific $CD8^+$ T cells observed in CMV seronegative recipients who received transplants from seropositive donors and who had not experienced CMV replication (Cwynarski *et al.*, 2001, Gandhi *et al.*, 2003a). Due to insufficient material, however, crossreactivity of tetramer binding T cells could not be substantiated in patient 7. Crossreactivity is not likely for patient 15 who did not show clinical signs of GvHD. CMV reactivation that was not detected at localised tissue sites may have induced proliferation of residual mature T cells of recipient origin in this patient. Reduced intensity conditioning in this patient resulted in 45 - 55 % chimaerism at the time of follow-up, which is consistent with the hypothesis that tetramer binding cells originated from mature recipient T cells.

3-3.5 Co-dominance of CMV specific CD8$^+$ T cells targeting different HLA/peptide combinations

In view of previous findings of HLA-B*0702/pp65 (417-426) restricted CMV pp65 specific CD8$^+$ T cell responses dominating HLA-A*0201/pp65 (495-503) restricted CMV pp65 specific CD8$^+$ T cell responses (Lacey *et al.*, 2003), results from this project were observed closely to detect any possible recurring dominance of a particular response in patients. In other studies CMV seropositive individuals expressing HLA-A*0201 and HLA-B*0702 demonstrate dominant responses restricted by the HLA-B*0702 but not all of these individuals lack responses restricted by HLA-A*0201 (Hebart *et al.*, 2002, Nastke *et al.*, 2005) suggesting preferential peptide presentation (compare section 1-7 from page 58 onwards) or preferential clonal proliferation rather than clonal deletion as the underlying mechanism for the observed dominance. Analysis of CMV seropositive patients simultaneously expressing several different HLA tissue types, for which tetramer reagents were available during this project, revealed no obvious dominance of either of the CMV specific CD8$^+$ T cells restricted by HLA-B35, HLA-A1 and HLA-A2 over each other. CMV specific CD8$^+$ T cells restricted by HLA-A24, however, seemed to be subdominant. All patients expressing HLA-A24 and one of the other HLA types simultaneously responded solely with CMV specific CD8$^+$ T cells restricted by the other HLA type. Previous studies suggested that the CMV pp65 (341-349) CD8$^+$ T cell epitope presented by HLA-A*2402 may not be immunodominant (Kondo *et al.*, 2003) due to the very low percentages (~0.1 %) of CD8$^+$ T cells targeting this epitope in healthy seropositive individuals (Kuzushima *et al.*, 2001). Similarly, low frequencies were observed in healthy individuals during this project (Table 3-4, page 206). First published observations of HLA-A*2402 restricted CMV pp65 (341-349) specific CD8$^+$ T cells in HSCT patients demonstrated frequencies of up to 2.85 % of CD8$^+$ T cells (Gondo *et al.*, 2004). Three patients studied here reconstituted maximum frequencies of 1.41 %, 5.00 % and 8.89 % HLA-A*2402 restricted CMV pp65 (341-349) specific cells amongst their CD8$^+$ T cells. These are possibly the highest frequencies of those cells that have been observed *ex vivo* yet. However, HLA-A24/pp65 specific CD8$^+$ T cells were present in only a fraction of patients expressing the HLA-A*2402 allele (3 of 12). The presence of HLA-A24/pp65 specific CD8$^+$ T cells in only a fraction of patients expressing the HLA-A*2402 allele may be one possible explanation for the suggestion

that this allele is one of the HLA alleles associated with an increased risk of CMV reactivation in the HSCT setting (Chen *et al.*, 2001).

Co-dominance of CMV specific $CD8^+$ T cells with targets other than HLA-A24/pp65 was observed amongst virus carriers. Figure 3-29 demonstrates similar frequencies of CMV specific $CD8^+$ T cells restricted by HLA-B35 *versus* HLA-A1 or HLA-A2 in patients expressing two of these HLA alleles simultaneously.

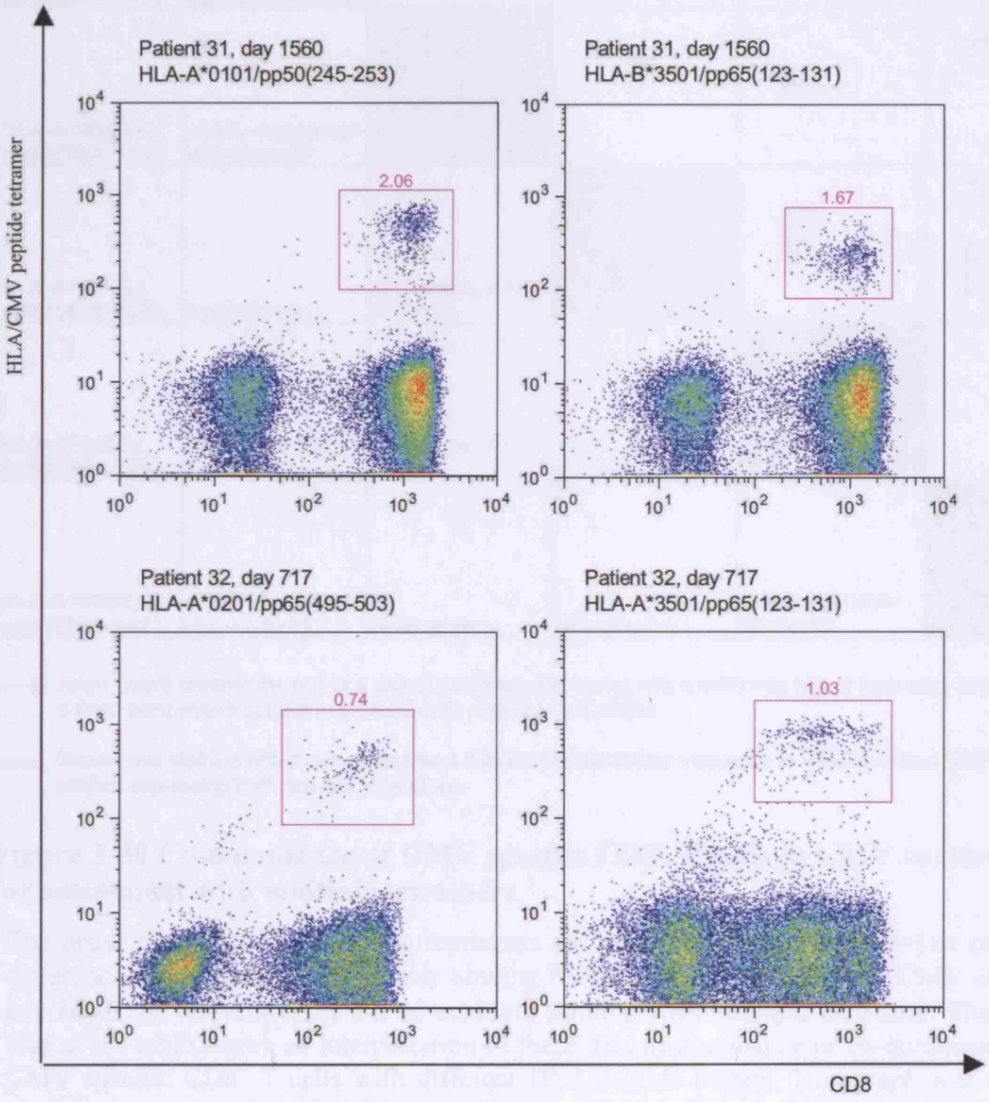
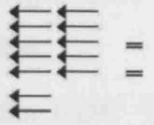
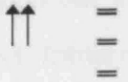
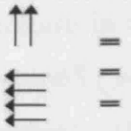


Figure 3-29 Co-dominance of CMV specific $CD8^+$ T cells restricted by HLA-B35, HLA-A1 or HLA-A2 in two representative patients

This representative example demonstrates tetramer stained PBMC from two patients. Flow cytometry plots show $CD3^+$ live lymphocytes sorted according to their surface expression of CD8 on the x-axis and staining with different HLA/CMV tetramer on the y-axis.

Figure 3-30 illustrates the relative frequencies of CMV specific $CD8^+$ T cells with different target specificities in the same virus carriers.

	HLA-A*0101/ pp50(245-253)	HLA-A*0101/ pp65(363-373)	HLA-A*0201/ pp65(405-503)	HLA-A*2402/ pp65(341-349)	HLA-B*3501/ pp65(123-131)
HLA-A*0101 / pp50(245-253)					
HLA-A*0101/ pp65(363-373)	mostly dominance HLA-A1/pp50				
HLA-A*0201/ pp65(405-503)	codominance	dominance HLA-A2/pp65			
HLA-A*2402/ pp65(341-349)	dominance HLA-A1/pp50	dominance HLA-A1/pp65	dominance HLA-A2/pp65		
HLA-B*3501/ pp65(123-131)	mostly dominance HLA-A1/pp50	codominance	codominance	dominance HLA-B35/pp65	

→ Arrow points towards the one of a pair of tetramers that bound with significantly higher frequency to PBMCs from a CMV seropositive subject expressing both relevant HLA alleles

= Represents staining with a pair of tetramers that bound with similar frequency to PBMCs from a CMV seropositive subject expressing both relevant HLA alleles

Figure 3-30 Co-dominance of CMV specific CD8$^+$ T cells in CMV carriers eligible for assessment with multiple tetramers

The upper right part of this figure represents each observation of similar (=) or considerably different (→) frequencies of tetramer binding PBMC that were observed in CMV carriers who expressed the relevant HLA alleles enabling staining with multiple tetramers. The lower left part of the table shows an interpretation of these data as dominance or co-dominance between CMV specific CD8$^+$ T cells with different HLA/peptide targets. This graph was drawn in a similar way as a graph by Lidehall and colleagues (Lidehall *et al.*, 2005).

Apart from the subdominance of HLA-A24/pp65 specific CD8$^+$ T cell responses described earlier, findings demonstrate no exclusive (sub)dominance of CMV specific CD8$^+$ T cells of one target specificity over others. In accordance with this co-dominance of CMV specific CD8$^+$ T cell epitopes was also described in other studies (Nastke *et al.*, 2005). HLA-A2/pp65 and HLA-B7/pp65 specific CD8$^+$ T cells may have been seen as dominant over other CMV specific CD8$^+$ T cells by some researchers in the field

previously. Results from Figure 3-30 challenge this view because frequencies of HLA-B35/pp65 specific cells substantially exceeded those of HLA-A2/pp65 specific cells in two patients (patients 1 and 2).

3-3.6 Revision of a single CMV epitope suggested to be presented by HLA-A1 and HLA-A24 alleles

A previous study suggested that $CD8^+$ T cell responses recognise the peptide CMV pp65 (341-349) presented not only by HLA-A*2402 (compare section 3-2.2) but also by HLA-A*0101 (Ghei *et al.*, 2005). That study measured IFNγ mRNA transcript production by cells that were peptide induced (*versus* unstimulated) for 3 hours *in vitro*. This peptide induction was performed after a 2-week sensitisation (cell culture in the presence of a 16mer comprising the sequence R**QYDPVAALF**FFDIDL or pp65 (341-349), and IL2) and subsequent culture in peptide-free medium overnight. This measurement was performed using cDNA for quantitative real time PCR. Additionally IFNγ protein release by cells (at 24, 48 and 72 hours after re-stimulation with pp65 (341-349), that was performed after 2-week sensitisation with the same peptide or R**QYDPVAALF**FFDIDL) was assessed by ELISA.

Studying the pattern of $CD8^+$ T cell responses to pp65 (341-349) presented by either HLA-A*2402 or HLA-A*0101 would be of interest because it has been suggested that significant differences (such as recruitment of distinct TCR repertoires and significant differences in the functional avidity of $CD8^+$ T cells) exist between $CD8^+$ T cells targeting identical epitopes but restricted by different HLA (Leslie *et al.*, 2006). However, the restriction of pp65 (341-349) by HLA-A*0101 was not confirmed in this study. The peptide CMV pp65 (341-349) refolded with HLA-A*2402 but not HLA-A*0101 *in vitro* (Figure 3-12, page 210) and no considerable IFNγ release of *ex vivo* PBMC from individuals expressing HLA-A*0101 (and not expressing HLA-A*2402) was observed using ELISpot assays (Figure 3-13, page 212).

3-3.7 Implications of findings and final conclusions

Cytomegalovirus disease has been recognised as a significant challenge in HSCT recipients. Patients are immunosuppressed profoundly in the early post-transplant period, and reactivation of CMV from latently infected cells remains a significant cause of morbidity and mortality.

Previous investigations demonstrated that CMV specific $CD8^+$ T cell numbers (inversely) correlate with viral replication and clinical status in patients. These mainly focused on enumeration of CMV specific $CD8^+$ T cell responses restricted by HLA-A2 and HLA-B7 (Aubert *et al.*, 2001, Cwynarski *et al.*, 2001, Engstrand *et al.*, 2000, Gillespie *et al.*, 2000, Gratama *et al.*, 2001, Komatsu *et al.*, 2003, Mohty *et al.*, 2004, Ozdemir *et al.*, 2002, Singhal *et al.*, 2000). This may have been for several reasons. Firstly, these alleles are amongst the most common HLA alleles in many ethnic groups. Secondly, their high numbers are easily detectable in patients and they were subsequently regarded as predominant. Thirdly, the generation of staining reagents for other CMV specific $CD8^+$ T cells responses such as those restricted by HLA-A24 and HLA-B35 can require some modification of the standard protocol (compare Chapter 2).

Tetramer based monitoring established that recovery of $1x10^7$ (Cwynarski *et al.*, 2001) to $2x10^7$ (Aubert *et al.*, 2001) HLA-A2/pp65 specific $CD8^+$ T cells/L of blood is associated with protection from CMV disease in patients.

The study described here aimed to compare the pattern of CMV specific $CD8^+$ T cell frequencies targeting other common HLA/peptide combinations in HSCT patients over time and to investigate the correlation of these cell frequencies with protection from CMV in patients.

To my knowledge, at the time of initiating this project this was the first study to conduct a longitudinal study of HLA-B35/pp65 specific $CD8^+$ T cell responses in the HSCT setting. $CD8^+$ T cells targeting pp65 (123-131) restricted by HLA-B*3501 were measured. Other studies previously enumerated $CD8^+$ T cells targeting pp65 (187-195) restricted by HLA-B*3503 (Wills *et al.*, 2002b) in healthy virus carriers or $CD8^+$ T cells targeting pp65 (188-195) restricted by HLA-B*3502 in HIV infected patients (Villacres *et al.*, 2003). $CD8^+$ T cells targeting pp65 (123-131) restricted by HLA-B*3501 have previously be monitored longitudinally in liver transplant patients (Hassan-Walker *et al.*, 2001). The occurrence of HLA-A1/pp50 specific (Lacey *et al.*, 2005) and HLA-A24/pp65 specific $CD8^+$ T cell responses (Gondo *et al.*, 2004, Morita *et al.*, 2005) were

demonstrated in the HSCT setting by other groups during the time of investigations for this project. However this study aimed to further these findings by more frequent analysis for determination of the level of responses correlating with protection from CMV in HSCT patients.

Findings from this study demonstrated that CMV specific $CD8^+$ T cells with different target specificities developed co-dominantly in HSCT patients. T cells were functional and, in contrast to previous reports (Ghei *et al.*, 2005), recognised CMV peptides including pp65 (341-349) in a highly HLA specific manner. Surprisingly CMV specific $CD8^+$ T cells were also detected in two recipients early post HSCT from CMV seronegative donors and it was suspected that these cells might be crossreactive in at least one of the two patients. It was found that CMV specific $CD8^+$ T cell levels above which patients appeared to be protected from subsequent CMV replication if $CD4^+$ T cells $\geq$160 cells/µl were present were significantly different depending on their target specificity.

Findings suggest an important role for HLA-B35/pp65 specific $CD8^+$ T cell responses. These responses inversely correlated with the ability to detect CMV reactivation at significantly lower levels than HLA-A1/pp50 and HLA-A2/pp65 specific $CD8^+$ T cell responses in HSCT patients. This suggests that HLA-B35/pp65 specific $CD8^+$ T cells may be of higher efficiency with fewer cell numbers required to protect patients from CMV replication than is the case for HLA-A1/pp50 or HLA-A2/pp65 specific $CD8^+$ T cells. HLA-A24/pp65 specific $CD8^+$ T cell responses also inversely correlated with the ability to detect CMV reactivation at lower levels than the latter two mentioned but were only observed in a minority of patients in this study and may be regarded as subdominant. Lower frequencies of HLA-B35/pp65 and HLA-A24/pp65 specific than HLA-A1/pp50 and HLA-A2/pp65 specific $CD8^+$ T cells were also observed in healthy volunteers.

These findings raise the question of how complex the total CMV specific $CD8^+$ T cell response may be. Table 3-12 lists an estimate of these proportions by the use of data shown earlier within this chapter but this time demonstrated as the ratio of the increase of CMV specific $CD8^+$ T cells and total $CD8^+$ T cells after CMV reactivation.

Patient	HLA-B35/pp65	HLA-A24/pp65	HLA-A1/pp50	HLA-A2/pp65
1	10.2 %			
2	0.9 %			0.8 %
4	0.5 %			
6	1.3 %			7.2 %
8	13.4 %			
9	8.5 %			
10 (e)		0.8 %		
10 (l)		37.7 %		
11		10.2 %		
12		1.0 %		
21 (e)			12.4 %	
21 (l)			2.1 %	
22			3.3 %	
23			2.1 %	2.2 %

Table 3-12 Estimated proportion of CMV specific $CD8^+$ T cells targeting different HLA/peptide combinations of the total CMV specific $CD8^+$ T cell response

Patients with significant numbers of tetramer binding cells after at least one CMV reactivation period are listed on the left (e: early reactivation period, l: late reactivation period). The increase of cells bound by different tetramers (listed on the top of the columns) is shown as a percentage of the increase of all $CD8^+$ T cells at the same time. The increase in cell numbers was measured from a time point immediately prior to CMV reactivation until the first peak of the response observed after that CMV reactivation. Due to a gap between sampling before and after CMV reactivation in patient 12 (Figure 3-7, page 198) the increase of the response has been measured from the time point of the lowest response post CMV reactivation onwards in this patient.

It should be noted that these values would represent the actual values of the proportion of the total CMV specific $CD8^+$ T cell response if the increase of total $CD8^+$ T cells post CMV reactivation was purely CMV specific. Due to cytokine induced bystander activity of some $CD8^+$ T cells, as an example, this may not be the case and therefore numbers may be an underestimate. However, findings shown in Table 3-12 suggest that CMV specific $CD8^+$ T cells measured within this project represent only a fraction of the total CMV specific $CD8^+$ T cell response in these patients.

$CD8^+$ T cells targeting different HLA/peptide combinations were seen to expand in parallel. Interestingly the pattern of these different responses was observed to vary slightly. For example in patient 6, HLA-A2/pp65 specific $CD8^+$ T cells peaked at an earlier time point than HLA-B35/pp65 specific $CD8^+$ T cells after CMV reactivation around day 56 post HSCT. In contrast in patient 2, HLA-B35/pp65 specific $CD8^+$ T cells peaked at an earlier time point than HLA-A2/pp65 specific $CD8^+$ T cells after CMV reactivation around day 549 post HSCT. In both patients the CMV specific $CD8^+$

T cells reaching their peak earlier are also of higher magnitude than CMV specific $CD8^+$ T cells reaching their peak later.

The composition of the total CMV specific $CD8^+$ T cell response appears to change over time. Table 3-12 shows an estimated 0.8 % of the total CMV specific $CD8^+$ T cell response to be targeting HLA-A24/pp65 after an early CMV reactivation (day 65) in patient 10. This response had increased to an estimated proportion of 37.7 % of the total CMV specific $CD8^+$ T cell response after a later CMV reactivation (day 89) in this patient. In contrast the HLA-A1/pp50 specific $CD8^+$ T cell response observed in patient 21 was estimated to have decreased from 12.4 % after an early CMV reactivation (day 153) to 2.1 % of the total CMV specific $CD8^+$ T cell response after a later CMV reactivation (day 438).

These observations and findings shown in Figure 3-30 (page 252) suggest that the total $CD8^+$ T cell response to CMV may be complex and multifaceted. The HLA-A2/pp65 specific $CD8^+$ T cell response may not be as universally immunodominant as previously thought. Attempts were undertaken to study the proportion of antigen specific $CD8^+$ T cells within the total CMV specific $CD8^+$ T cells that are restricted by a single HLA allomorph. Therefore it was aimed to use CMV permissive - and HLA class I deficient cells lines. Transfection with a single HLA class I protein would then allow the study of T cells responses (restricted to that HLA protein) towards cell lines that were either infected with CMV, infected with a vector containing a specific CMV derived protein or pulsed with CMV epitope peptides. Since primary trophoblasts are known to be CMV permissive (Fisher *et al.*, 2000, Maidji *et al.*, 2002) and the syncytiotrophoblast-like human choriocarcinoma cell line JAR, a first trimester trophoblast cell model, is deficient for classical major HLA class I molecules (King *et al.*, 1996), JAR was chosen as one of the cell line candidates for this experiment. This cell line was successfully transfected with HLA class I and infected with influenza A previously (Gobin *et al.*, 1997). Unfortunately, this and other cell lines tested could not be productively infected with two available CMV laboratory strains in this study. An example of the infection trials is shown in Figure 3-31. It may be that CMV infects only cytotrophoblasts but not syncytiotrohoblasts or that the cell line was non-productively infected as was demonstrated for trophoblast cell lines infected with other viruses previously (Liu *et al.*, 1995b, Zachar and Ebbesen, 1991). Alternatively the fibroblast adapted virus strains used, may not be suitable for the infection of the epithelial-like cell line used.

Limited accessibility of other virus strains and of primary trophoblasts as well as the expertise required for handling of those, made it not feasible to use them in this study. Therefore experiments were not pursued further. However, future experiments could use a different combination of cell line and virus strain similar to that used in a recent report (LaMarca *et al.*, 2006).

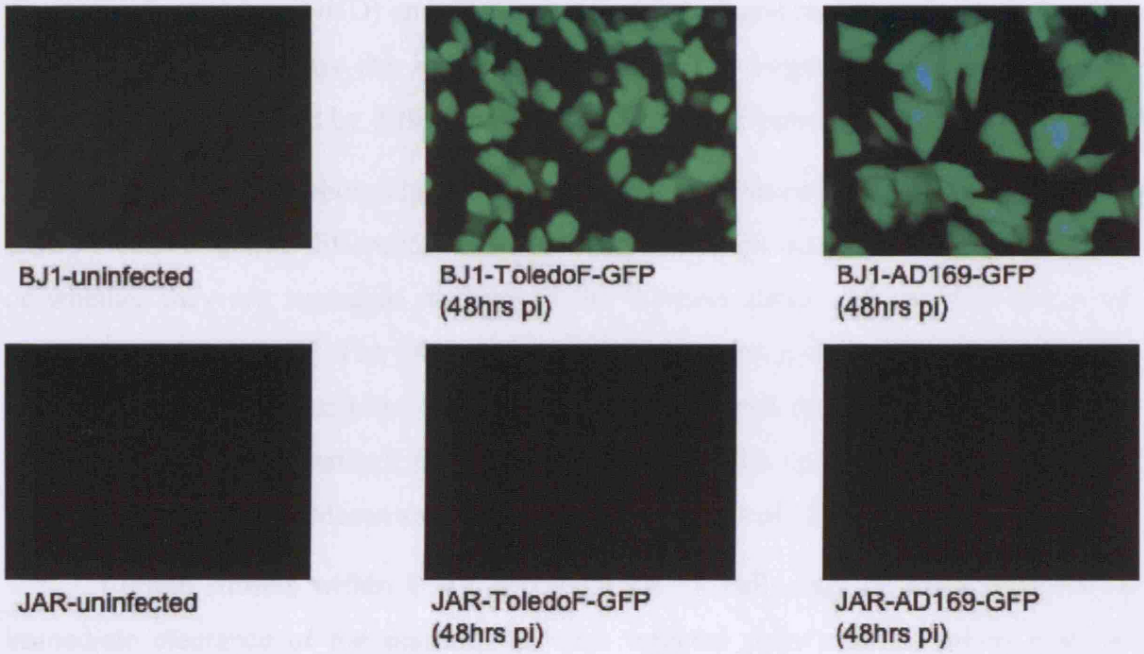


Figure 3-31 CMV infection of cell lines

This figure shows fluorescent microphotographs of the hTERT-BJ1 control fibroblast cell line on the top and of the JAR cell line on the bottom. Cells on the left were left uninfected; cells in the middle are shown 48 hours post infection with the enhanced green fluorescent (EGFP) recombinant Toledo-F CMV strain; cells on the right are shown 48 hours post infection with the EGFP recombinant AD169 CMV strain. In both CMV strains EGFP was used as a reported gene under the transcriptional control of the ß2.7 promoter (McSharry *et al.*, 2003). The ß2.7 is the most abundantly transcribed early gene from the CMV genome with maximal amplification between 8 and 14 hours post infection and remaining actively transcribed during the replication cycle (McDonough *et al.*, 1985). Expression of EGFP can be seen under UV light, where it emits green light. Observations after 72 hours were similar (not shown).

As the staining reagents for HLA-B35/pp65 and HLA-A24/pp65 specific $CD8^+$ T cells are now readily available commercially and their measurement was reported to be feasible for routine monitoring (Buxbaum *et al.*, 2006), future investigations may hopefully include responses to these and new targets (such as new pp65 epitopes restricted by HLA-A*2402 and HLA-B*3501 described in 2007) that are constantly discovered (Burrows *et al.*, 2007, Khan *et al.*, 2007). A study funded by Beckman Coulter, one of the companies offering tetramers commercially, recently investigated CMV specific $CD8^+$ T cell responses to a variety the targets. Responses were monitored

in HSCT patients and a brief, one-page long, report confirmed detectable portions of tetramer binding cells for all tetramers without going into much detail (Gratama *et al.*, 2004). The authors of that study reported dim staining for HLA-A24/pp65, which was not observed with in-house made tetramer in this study. Furthermore they reported an inverse correlation between three clinical factors (the risks of recurrent viraemia, CMV disease and extensive GvHD) and CMV specific $CD8^+$ T cell responses in patients. That report therefore confirms the importance of responses targeting a variety of CMV specific targets restricted by different HLA types in HSCT patients.

It remains to be shown in future cell transfer experiments whether CMV specific $CD8^+$ T cells targeting different HLA/peptide combinations actually mediate protection or whether they are surrogate markers of the immune status and thus the ability of patients to combat CMV. The proportion of these cells within the total of CMV specific $CD8^+$ T cells that are restricted by a single HLA allomorph could be studied using the extravillus cytotrophoblast cell line SGHPL-4 infected with the CMV strain RVdlMwt-GFP as described by LaMarca and colleagues (LaMarca *et al.*, 2006) in the future.

Certain subsets within CMV specific $CD8^+$ T cells may be most effective at immediate clearance of the majority of virus infected cells whereas others may be necessary for long term control of CMV replication from latently infected cells. This may explain the phenotypic diversity that can be observed within CMV specific $CD8^+$ T cells (Figure 3-32). This heterogeneity has also been observed within CMV specific $CD8^+$ T cells of the same clonal origin (Weekes *et al.*, 1999c, Wills *et al.*, 2002b).

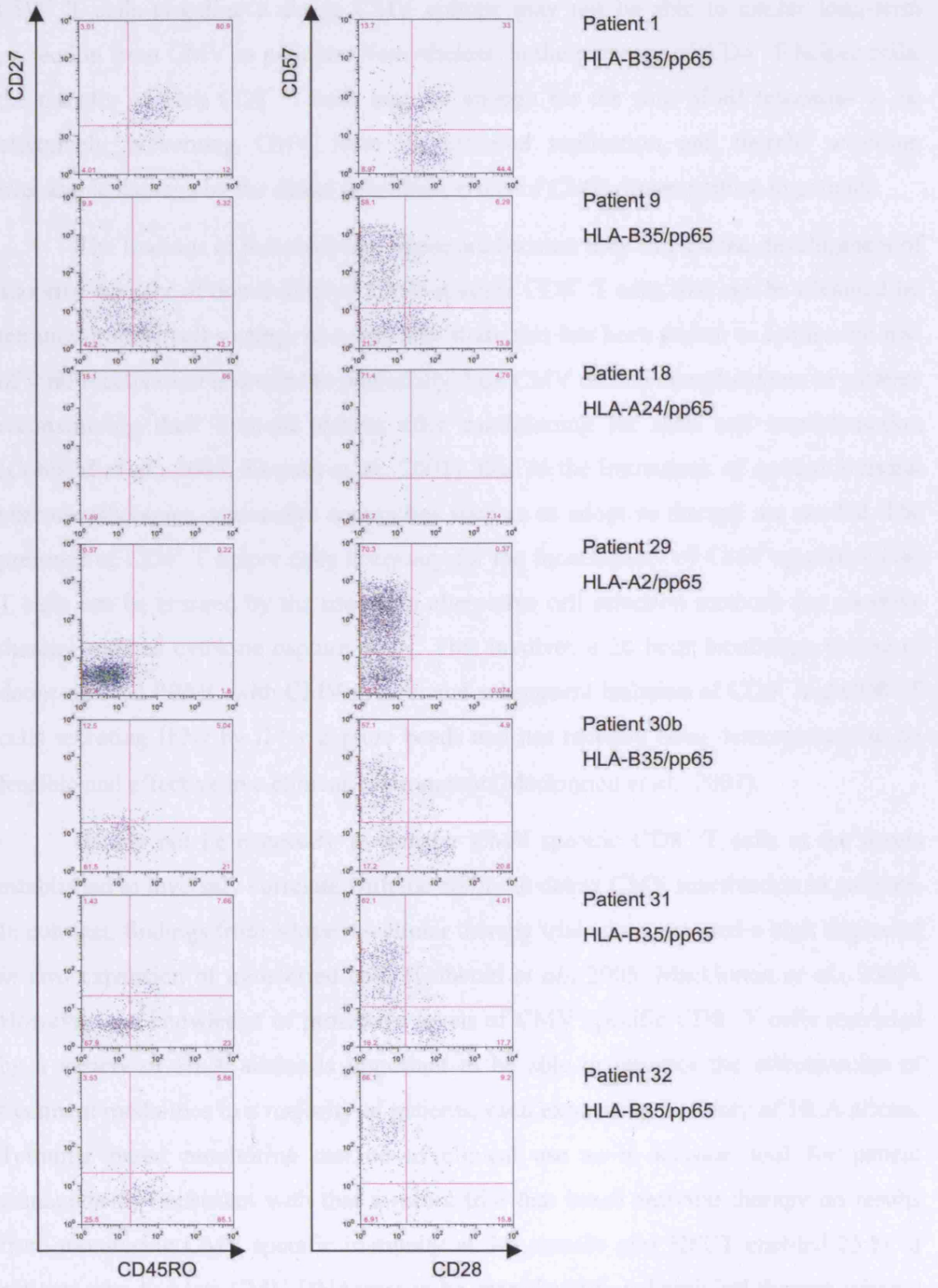


Figure 3-32 Heterogeneous phenotypes of CMV specific $CD8^+$ T cells

Flow cytometry plots represent $CD8^+$ T cells that were bound by tetramers listed on the right. Staining with CD27 and CD45RO on the left demonstrates various differentiation stages of CMV specific memory $CD8^+$ T cells. Staining on the right demonstrates the proportions of CMV specific $CD8^+$ T cells that are $CD57^+$ $CD28^-$, a phenotype that was previously associated with terminally differentiated non-proliferating effector T cells (Kern *et al.*, 1996) but was since reported to represent a highly differentiated T cell subset with unique functionality (including the capability of proliferation) late after activation (Chong *et al.*, 2008).

$CD8^+$ T cells targeting a single CMV epitope may not be able to confer long-term protection from CMV in patients. Nevertheless, in the presence of $CD4^+$ T helper cells, the transfer of such $CD8^+$ T cells may be enough for the sum of all responses to be effectively preventing CMV from uncontrolled replication and thereby avoiding irreparable damage by the direct or indirect effect of CMV dissemination in patients.

The findings of this study are important because they can aid the development of adoptive transfer of donor-derived CMV-specific $CD8^+$ T cells that can be obtained by tetramer guided cell sorting. In a previous study this has been shown to reduce the rate of viral reactivation and combat potentially fatal CMV related complications in patients reconstituting their immune system after conditioning for stem cell transplantation (Cobbold *et al.*, 2005, Keenan *et al.*, 2001). Due to the limitations of current antiviral pharmacotherapies, alternative approaches such as an adoptive therapy are needed. The presence of $CD4^+$ T helper cells necessary for the functionality of CMV specific $CD8^+$ T cells can be ensured by the use of an alternative cell selection methods for adoptive therapy such as cytokine capture assay. This involves a 20 hour incubation period of donor-derived PBMC with CMV protein and subsequent isolation of $CD4^+$ and $CD8^+$ T cells secreting IFNγ by IFNγ capture beads and has recently been demonstrated to be feasible and effective in a clinical environment (Mackinnon *et al.*, 2007).

It may not be necessary to transfer CMV specific $CD8^+$ T cells at the levels established to inversely correlate with the ability to detect CMV reactivation in patients. In contrast, findings from adoptive cellular therapy trials demonstrated a high degree of *in vivo* expansion of transferred cells (Cobbold *et al.*, 2005, Mackinnon *et al.*, 2007). However, the knowledge of protective levels of CMV specific $CD8^+$ T cells restricted by a variety of HLA alleles is important to be able to monitor the effectiveness of treatment modalities in a majority of patients, each expressing a variety of HLA alleles. Tetramer based monitoring can be of clinical use as a decision tool for patient management. Consistent with that a recent trial that based antiviral therapy on results from monitoring CMV specific immunity at 3-6 months post HSCT enabled 25 % of patients who had late CMV DNAemia to be spared additional antiviral therapy without development of CMV disease. It was suggested that this strategy may allow more targeted use of treatment to patients at highest risk of developing CMV disease (Avetisyan *et al.*, 2007).

CHAPTER 4 CLONOTYPIC ANALYSIS OF *EX VIVO* $CD8^+$ T CELLS WITH DIFFERENT HLA/CMV PEPTIDE TARGETS

4-1 Introduction

The previous chapter described the measurement of *ex vivo* CMV specific $CD8^+$ T cell responses with different HLA/CMV peptide target specificities and their functionality in HSCT patients. Findings demonstrated that the median number of CMV specific $CD8^+$ T cells, which inversely correlated with the ability to detect CMV reactivation in patients was significantly different for different HLA/CMV peptide targets, which has important clinical implications.

This chapter describes the clonality of cells that were quantified in the previous chapter. Therefore the diversity of TCR used by the different CMV specific $CD8^+$ T cells was analysed. Some viral infections are known to generate T cells with public TCR usage (refer to section 1-8.2). Focused TCR usage was also shown for HLA-A*0201/CMVpp65(495-503) and HLA-B*0702/CMVpp65(417-426) (subsequently abbreviated as HLA-A2/pp65 and HLA-B7/pp65 respectively) specific $CD8^+$ T cells previously. Spectratyping and subsequent bacterial cloning of these cells demonstrated the use of a restricted number of TCR Vß families without conservation of CDR3 lengths or junctional motifs when donor PBMC were cultured with autologous DC and CMV antigen *in vitro* (Peggs *et al.*, 2002). CMV specific $CD8^+$ T cells derived from limiting dilution assay after stimulation with CMV infected fibroblasts also used a restricted number of Vß families as demonstrated by flow cytometry with Vß specific antibodies (Wills *et al.*, 1996). It has been suggested recently that the underlying mechanism for this TCR repertoire focusing may be the selection of high affinity clones into the memory $CD8^+$ T cell pool (Day *et al.*, 2007).

Although recent publications argue against it (as will be discussed in section 4-3), it is possible that TCR CDR3 diversity may be underrepresented during the analysis of sequences of T cell clones that were generated by cell expansion, because only clones able to proliferate *in vitro* may be detected. TCR analysis reported prior to initiation of this project was limited in that it was only performed for CMV specific T cells restricted by HLA-A2, HLA-B7 and HLA-B44 (Weekes *et al.*, 1999b, Peggs *et al.*, 2002).

This study aimed to compare the diversity of TCRs used by *ex vivo* CMV specific $CD8^+$ T cells with different HLA/CMV peptide target specificities. *Ex vivo* TCR analysis may be difficult in healthy virus carriers due to low frequencies of CMV specific $CD8^+$ T cells but it is possible in patients who expand these T cells after CMV reactivation post HSCT. TCR diversity of cells with a wide range of target specificities was assessed by spectratyping. The basics and examples of the application of this method were explained in section 1-8.2.

Spectratyping results can be illustrated in histograms representing peaks of fluorescent PCR products separated according to size. Each peak represents a set of T cell receptors bearing the same CDR3 length. The deletion and addition of nucleotides during rearrangement of T cell receptors is random. Therefore, for any Vß family, an unperturbed polyclonal repertoire such as that of cord blood is represented by a set of peaks distributed in a Gaussian pattern, with the most frequent CDR3 length situated in the centre (Garderet *et al.*, 1998). Clonal expansion results in changes in the relative peak heights. Skewing of the Gaussian distribution can be observed with increasing age, due to antigenic stimulation. Contraction of the T cell repertoire after immunosuppression can also lead to iatrogenic gaps in the spectratype. In both cases, oligoclonality is indicated by predominance of only a few size classes in a spectratype (Gorski *et al.*, 1994, Pannetier *et al.*, 1995).

Findings from analysing the clonality of $CD8^+$ T cells with different HLA/CMV peptide target specificities may help to further the knowledge of which cells may be most effective in patients. The virus may easily escape a monoclonal T cell response by production of antagonist peptide. It is, however, unlikely that such peptides are produced for all TCRs of a polyclonal response.

Knowledge of the clonality of CMV specific $CD8^+$ T cells may be useful for the development of treatment modalities using TCR gene transfer. The knowledge of TCRs used by $CD8^+$ T cells targeting different CMV antigens may be of clinical use for enhancing the detection during the monitoring of CMV responses in high-risk immunodeficient patients by the use of clonotypic probes. Furthermore the clonality of responses has an impact on patients beyond their protection against CMV related disease development. This includes overall narrowing of the TCR repertoire resulting in accelerated immune ageing in patients.

Section 4-2.1 within this chapter first describes what challenges were experienced during the spectratyping of small numbers of cells and how these were

overcome. Findings from analysing the TCR diversity of CMV specific $CD8^+$ T cells are subsequently shown in separate smaller sub-sections within section 4-2.2, sorted by the HLA/peptide combinations targeted. Potential artefacts from this analysis and confirmatory experiments are described in sections 4-2.3 and 4-2.4. Section 4-2.5 then demonstrates bacterial cloning and sequencing of TCRs from 4 Vß families, 2 of these each derived from HLA-A24/pp65 and HLA-B35/pp65 specific $CD8^+$ T cells. These were performed to assess the level of heterogeneity within the dominant CDR3 size classes observed by spectratyping. The impact of findings from these experiments is then discussed in section 4-3.

4-2 Results

This section describes the experimental estimate of TCR diversity of *ex vivo* CMV specific T cell responses in HSCT patients. The analysis was focused on $CD8^+$ α/β T cells targeting the different HLA/CMV peptide combinations investigated in Chapter 3.

4-2.1 Preparation of *ex vivo* CMV specific $CD8^+$ T cells for TCR Vß analysis

TCR CDR3 diversity may be underrepresented during the analysis of sequences of T cell clones that were generated by expansion in response to CMV antigen, because only clones able to proliferate *in vitro* may be detected with this method. It was therefore decided to analyse the TCR diversity of CMV specific $CD8^+$ T cells directly *ex vivo* using cDNA from sorted tetramer binding cells for PCR with TCR Vß family specific primers. Some products were used further for bacterial cloning and sequencing with results shown in section 4-2.5 from page 298 onwards.

To estimate the TCR diversity of *ex vivo* CMV specific $CD8^+$ T cells, PBMC stained with HLA/CMV peptide tetramer were sorted into the cell fraction that bound to tetramer and the remaining fraction of cells that did not bind to tetramer. RNA was then extracted from sorted cells followed by reverse transcription PCR to obtain cDNA. PCR amplification of the TCR CDR3 region from cDNA was performed and PCR products of different lengths were separated by gel/capillary electrophoresis.

Without *in vitro* expansion, numbers of sorted tetramer binding cells were initially small (often <3,000). Therefore several experimental steps needed extensive optimisation to achieve successful TCR Vß analysis of *ex vivo* CMV specific $CD8^+$ T cells, which is described below.

4-2.1.1 Optimisation of sorting efficiency of CMV specific CD8$^+$ T cells

PBMC stained with PE (or APC) labelled tetramer were initially sorted with anti-PE (or anti-APC) magnetic beads over two consecutive MACS columns with a representative result illustrated in Figure 4-1.

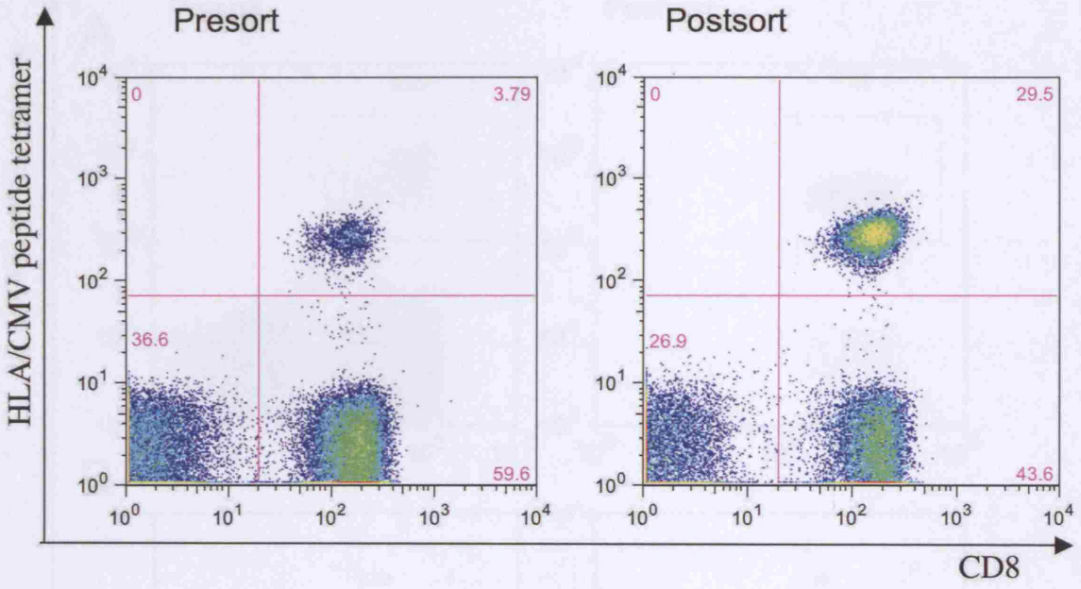


Figure 4-1 One-step sorting of tetramer stained cells using magnetic beads

This figure demonstrates a representative example of tetramer sorted PBMC (patient 38). Flow cytometry plots represent CD3$^+$ live lymphocytes with staining profiles before bead sorting shown on the left and after bead sorting on the right. Numbers represent the cells within each quadrant as a percentage of total cells in that plot.

This sorting approach resulted in very poor purity of sorted cells (70 % contamination of tetramer binding cells in the representative example shown). This was not adequate for further molecular analysing of these cells.

To achieve higher purity of sorted cells, a 2-step approach was then adopted. CD8$^+$ T cells were enriched in a first step by depletion of non-CD8$^+$ T cells with an antibody cocktail described in section 2-6.1, then stained with tetramer and sorted as described. However, the purity of sorted cells was highly dependent on their frequency within the original cell population, an observation also made by others (Bodinier *et al.*, 2000). Two representative cell sorting examples are shown in Figure 4-2 demonstrating higher purity of sorted cells that were present at high frequency in the original population (panels A) than of cells that were present at low frequency in the original population (panels B). This may be due to less saturation of beads during the binding of magnetic beads to cells with low numbers of specific cells than during the binding to

cells with high numbers of specific cells. This may, in turn, result in higher carry over of non-specific cells during the sorting of cells with low numbers of specific cells. Alternatively, the low purity of sorted cells may have resulted from a fixed background staining by tetramers leading to a lower signal to noise ratio during tetramer staining of low numbers of specific cells.

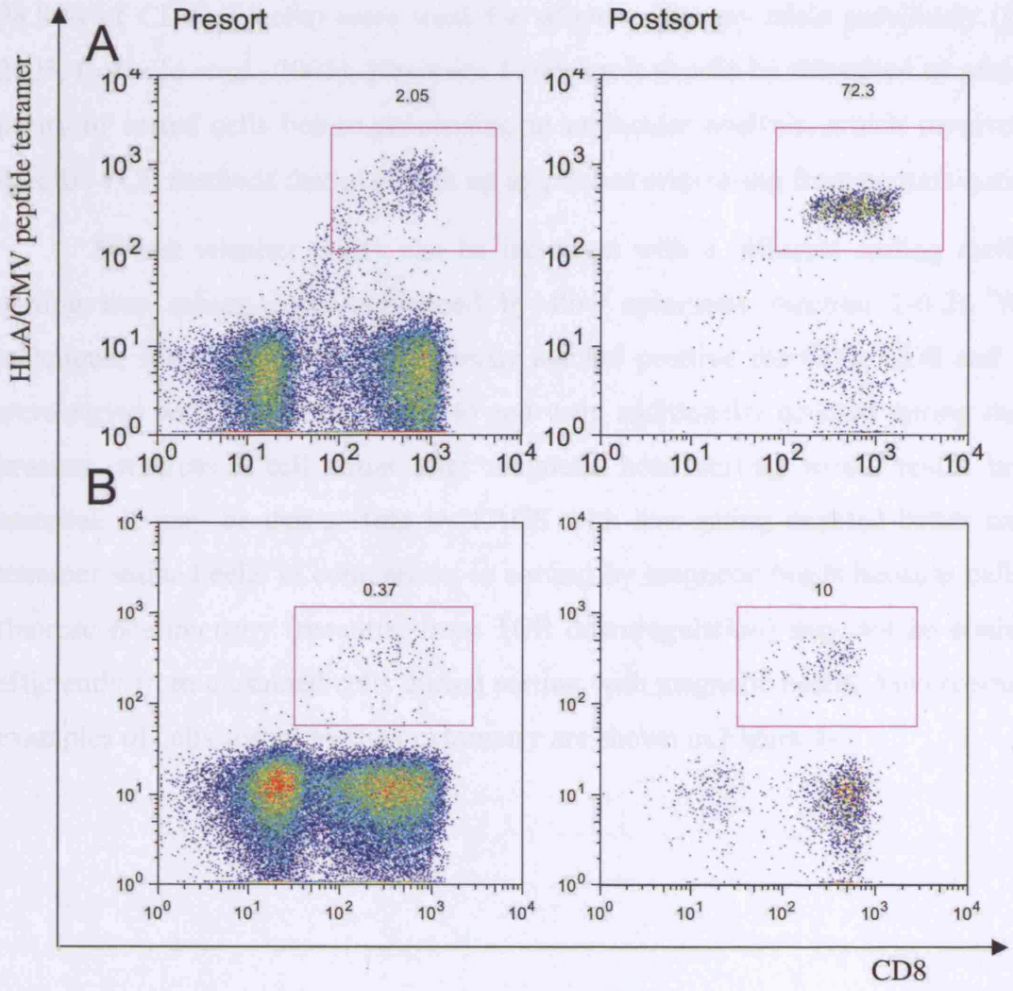


Figure 4-2 Two-step sorting of tetramer stained cells using magnetic beads

Tetramer stained peripheral blood cells from two individuals (A: patient 38; B: patient 4) are shown. Flow cytometry plots represent $CD3^+$ live lymphocytes with staining profiles before bead sorting shown on the left and after bead sorting on the right. Numbers in the upper right corner of the plots represent the cells shown in gates as a percentage of total cells in that plot.

Representative examples shown in Figure 4-2 are similar to findings reported by Bodinier and colleagues. In this study, specific cells with a frequency of 0.4 % in the original population were enriched to 10.0 % after sorting whereas specific cells with a frequency of 2.1 % in the original population were sorted to a purity of 72.3 % (Figure 4-2). In comparison, specific cells with a frequency of 0.2 % in the original population were enriched to 8.6 % after sorting whereas specific cells with a frequency of 1.4 % in

the original population were sorted to a purity of 75.9 % in the study by Bodinier and colleagues (Bodinier *et al.*, 2000).

Sorted cells of high purity were not obtained with either the 1-step or the 2-step approach using magnetic beads from either StemCell Technologies or Miltenyi Biotech. Magnetically sorted cells of purities as low as 10 % of $CD3^+$ T cells (corresponding to 98.8 % of $CD8^+$ T cells) were used for adoptive therapy trials previously (Cobbold, 2005, Cobbold *et al.*, 2005). However, I felt that it should be attempted to achieve high purity of sorted cells before proceeding to molecular analysis, which involves highly specific PCR methods that may pick up amplicons originating from contaminating cells.

To test whether purity can be increased with a different sorting method, cell sorting was subsequently performed by flow cytometry (section 2-6.2). With this technique, live cells that simultaneously stained positive for CD3, CD8 and tetramer were sorted with high purity (>90 %) and were additionally counted during the sorting process, whereas a cell count after magnetic bead sorting would result in loss of material. It may be that sorting by FACS with live gating enabled better capture of tetramer stained cells in comparison to sorting by magnetic beads because cells of low fluorescence intensity (resulting from TCR downregulation) may not be distinguished efficiently from unstained cells during sorting with magnetic beads. Two representative examples of cells sorted by flow cytometry are shown in Figure 4-3.

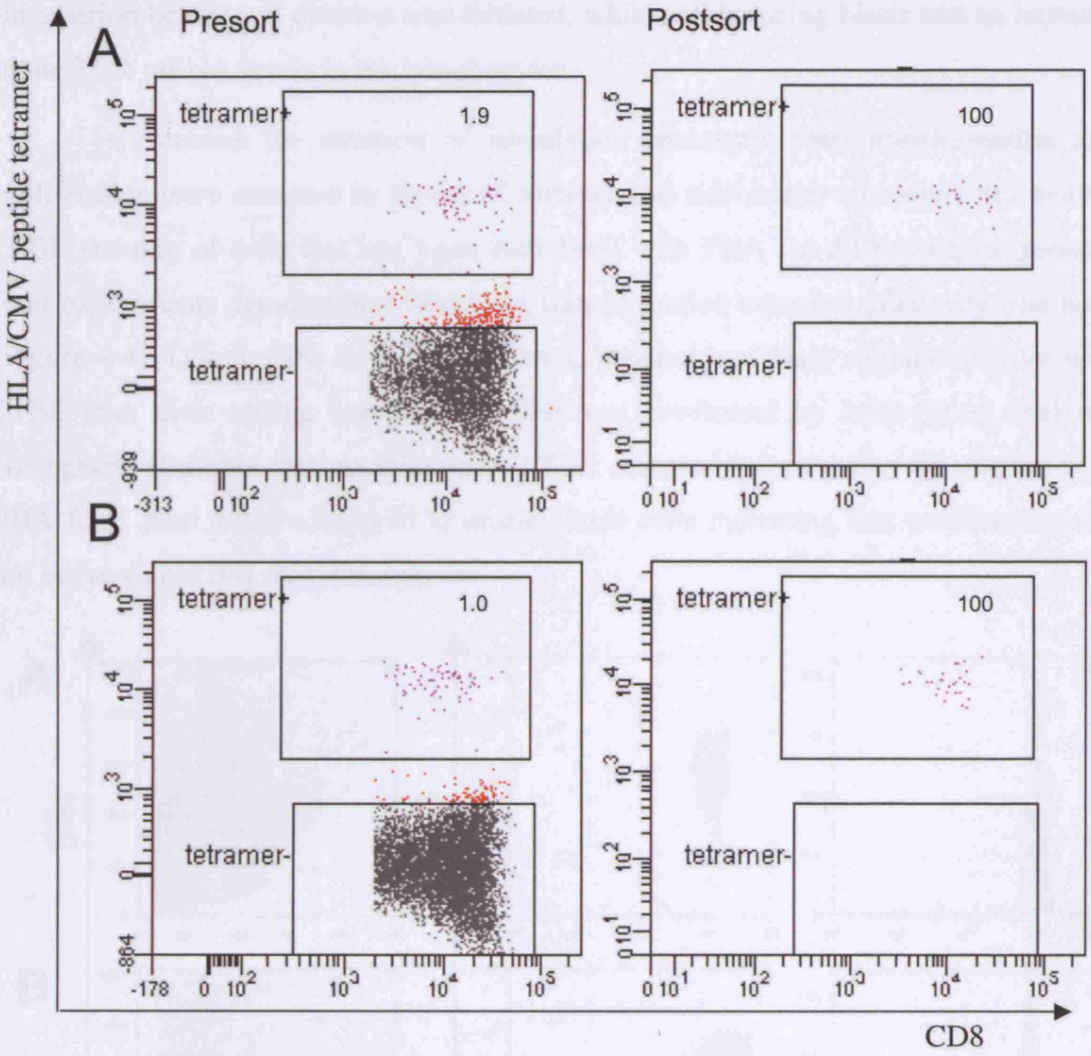


Figure 4-3 Sorting of tetramer stained cells using flow cytometry

Plots illustrate tetramer stained PBMC from two individuals (A: patient 22, HLA-A*0101/pp50 tetramer staining; B: patient 34, HLA-A*0101/pp65 tetramer staining). $CD3^+$ $CD8^+$ live cells are demonstrated with staining profiles before sorting shown on the left and staining profiles after bead sorting shown on the right. Numbers in the upper right corner of the plots represent the cells shown in gates as a percentage of total $CD8^+$ T cells.

4-2.1.2 Optimisation of mRNA yields by phytohaemagglutinin stimulation of T cells and choice of more efficient cDNA production methods

Sorting of *ex vivo* CMV specific $CD8^+$ T cells resulted in low yields of cells. To be able to analyse the TCR repertoire of these cells, a short stimulation of cells was performed to boost TCR mRNA levels. The rational behind this approach is that TCR transcription is up-regulated during T cell activation, which can be achieved by phytohaemagglutinin (PHA) or phorbol esters (Wotton *et al.*, 1993). To avoid artefacts introduced by differential proliferation of individual clones, it was intended to limit stimulation to a

time period before cell division was initiated, while still inducing blasts and an increase in the TCR mRNA levels in the lymphocytes.

To establish the duration of stimulation necessary, blast transformation and proliferation were assessed by means of forward and side scatter characteristics and by CFSE staining of cells that had been stimulated with PHA for different time periods. First experiments demonstrated that blast transformation occurred after only one hour (Figure 4-4). Due to their larger surface area, lymphoblasts stain slightly brighter with CFSE than their resting counterparts. This was confirmed by back-gating analysis. Histograms illustrate that no dilution of CFSE occurred in cells after stimulation with PHA for 1 hour when compared to unstimulated cells indicating that proliferation did not occur within this short time period.

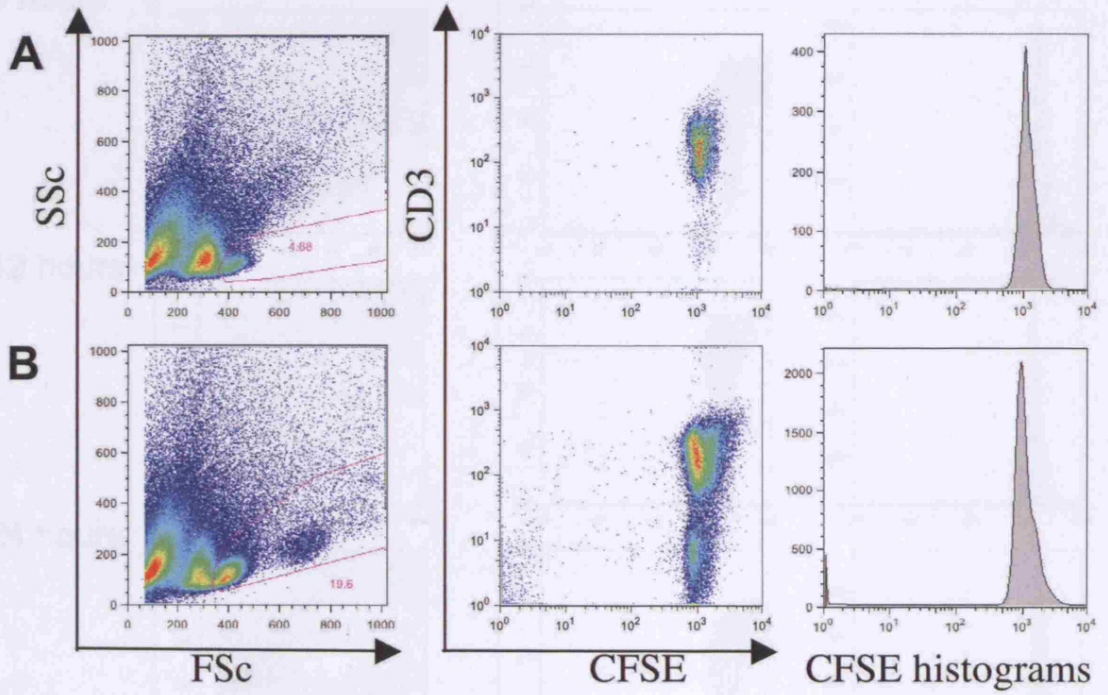
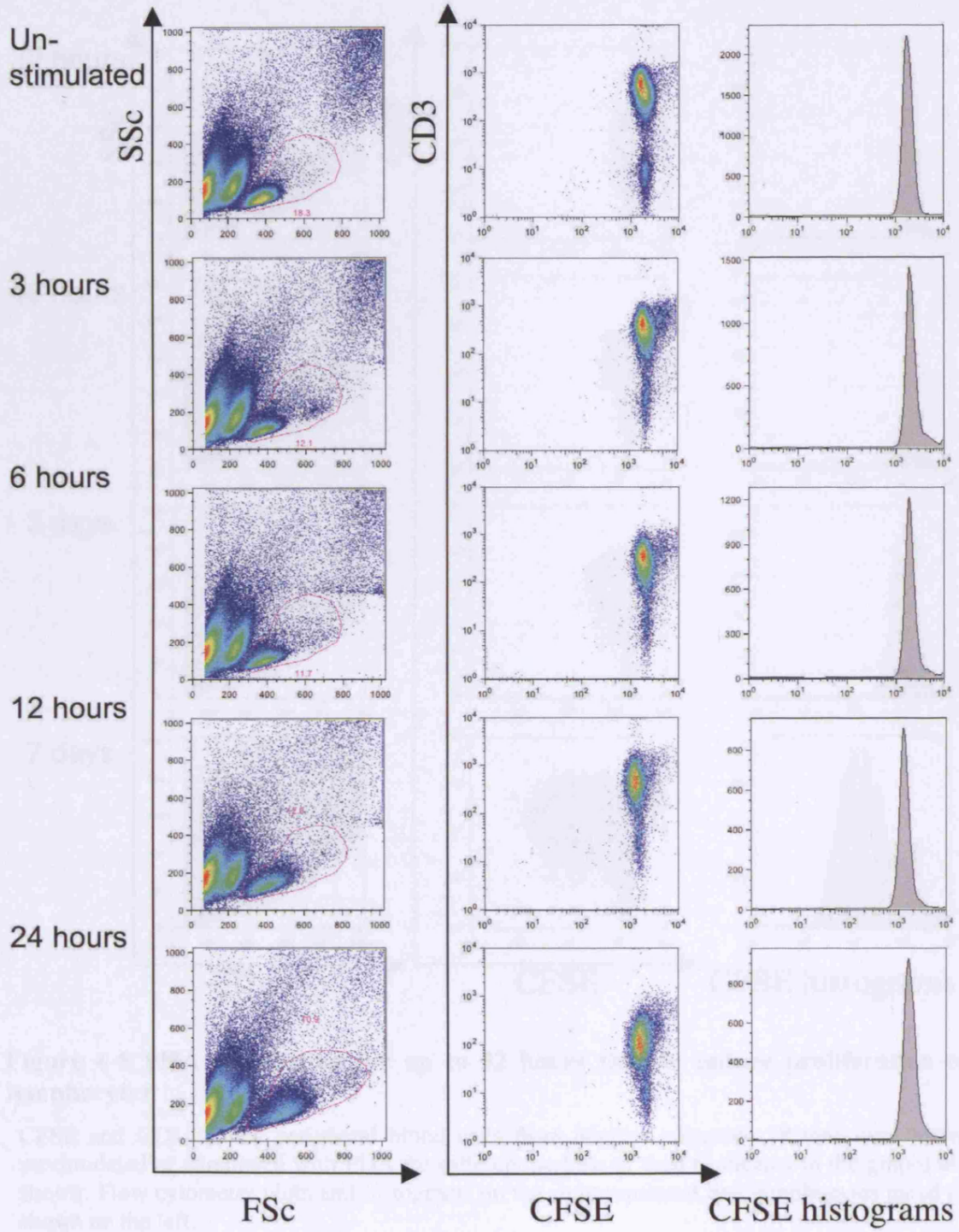


Figure 4-4 PHA stimulation induces lymphocyte blasts within 1 hour

CFSE and CD3 stained peripheral blood cells from healthy volunteer 17 that were either unstimulated (A) or stimulated with PHA for 1 hour (B) are shown. Flow cytometry plots and histograms on the right represent live lymphocytes gated as shown on the left.

No proliferation after PHA stimulation for up to 20 hours was observed in initial experiments. Therefore the use of longer time periods was investigated. Results can be seen in Figure 4-5. These were obtained from duplicate samples, each stained with CFSE and stimulated with PHA for a fixed period of time before CD3 staining and assessment by flow cytometry.


Un-stimulated
3 hours
6 hours
12 hours
24 hours
SSc
CD3
FSc
CFSE
CFSE histograms

(Continued)

(Continued)

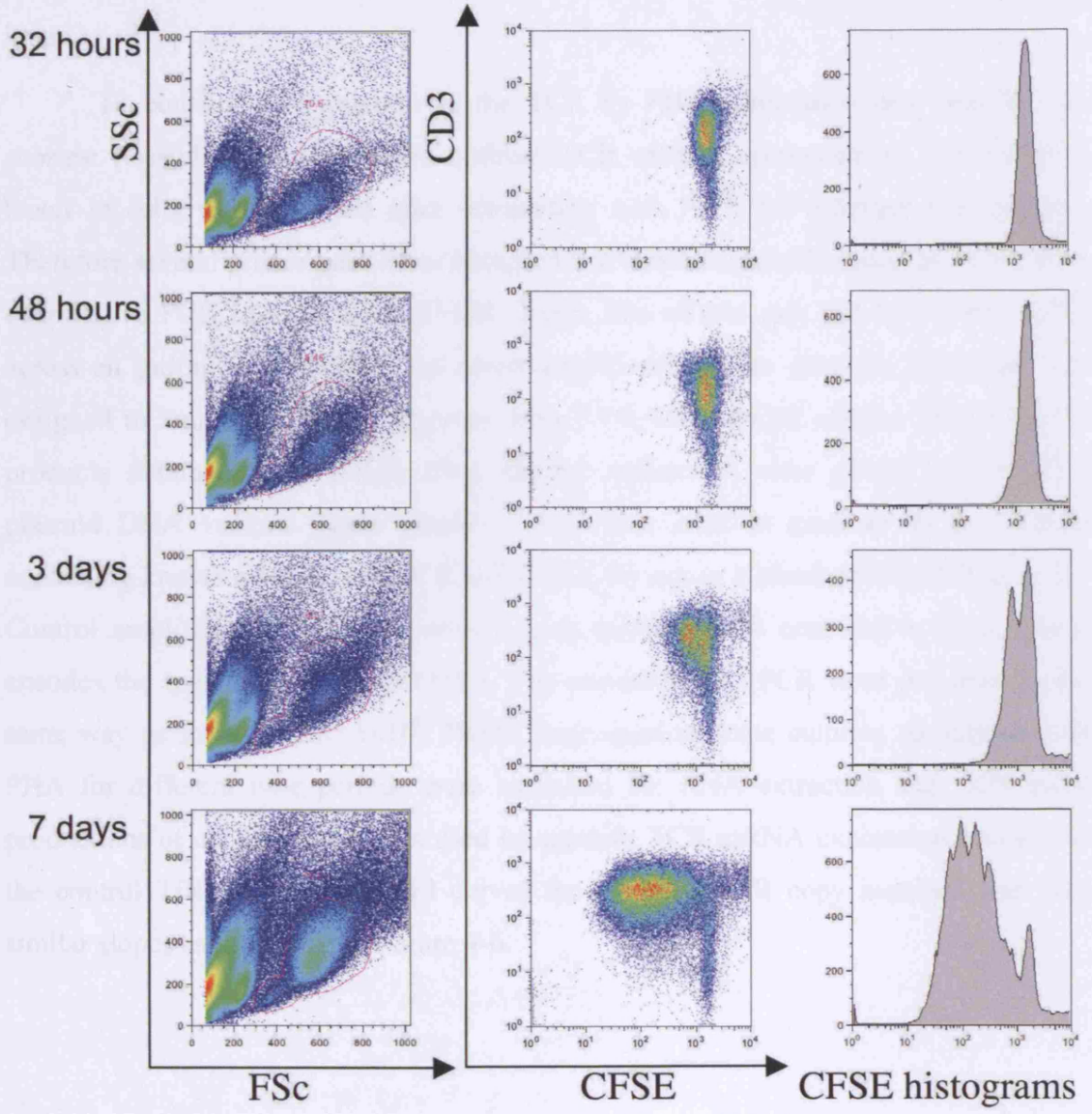


Figure 4-5 PHA stimulation for up to 32 hours fails to induce proliferation of lymphocytes

CFSE and CD3 stained peripheral blood cells from healthy volunteer 18 that were either unstimulated or stimulated with PHA for different periods of time (indicated in the graph) are shown. Flow cytometry plots and histograms on the right represent live lymphocytes gated as shown on the left.

All cells were left in culture for the same duration of time (7 days) during this experiment to adjust for gradual loss of CFSE over time (PHA stimulant was added at different times before harvest). This may explain why blasts shown in Figure 4-5 are less prominent than those shown in Figure 4-4, which were left in culture for less than 2 days. Daughter cells demonstrating lower CFSE staining ($<10^3$ fluorescence units) than parental cells are first visible after 48 hours of PHA stimulation (Figure 4-5). Results

from this experiment demonstrate that PHA stimulation induced blast formation of cells after the shortest stimulation period (2 hours) in the absence of proliferation for up to 32 hours.

To confirm up-regulation of the TCR by PHA stimulation and establish the shortest possible time to achieve optimal TCR mRNA up-regulation, TCR mRNA levels in cells were assessed after stimulation with PHA for different time periods. Therefore several primer pairs were designed and tested for amplification of TCR Cß by quantitative PCR (qPCR) using SYBR Green. The chosen pair had one primer lying across an intron/exon boundary to avoid amplification from genomic DNA and was designed to amplify a 195 bp fragment from TCR Cß1 or Cß2 cDNA. TCR Cß PCR products obtained from cDNA from healthy volunteers were cloned into pCR2.1 plasmid DNA vectors. These plasmids were then used to generate TCR dilutions containing known quantities of TCR molecules, for use as a standard in qPCR analysis. Control amplifications were performed using primers for a constitutive gene, which encodes the transferrin receptor (TfR). TfR standards for qPCR were prepared in the same way as for the TCR. $1x10^6$ PBMC from quadruplicate cultures stimulated with PHA for different time periods were harvested for RNA extraction and subsequent productions of cDNA, which was used to quantify TCR mRNA expression along with the control TfR mRNA. Standard curves for TCR and TfR copy numbers had very similar slopes as illustrated in Figure 4-6.

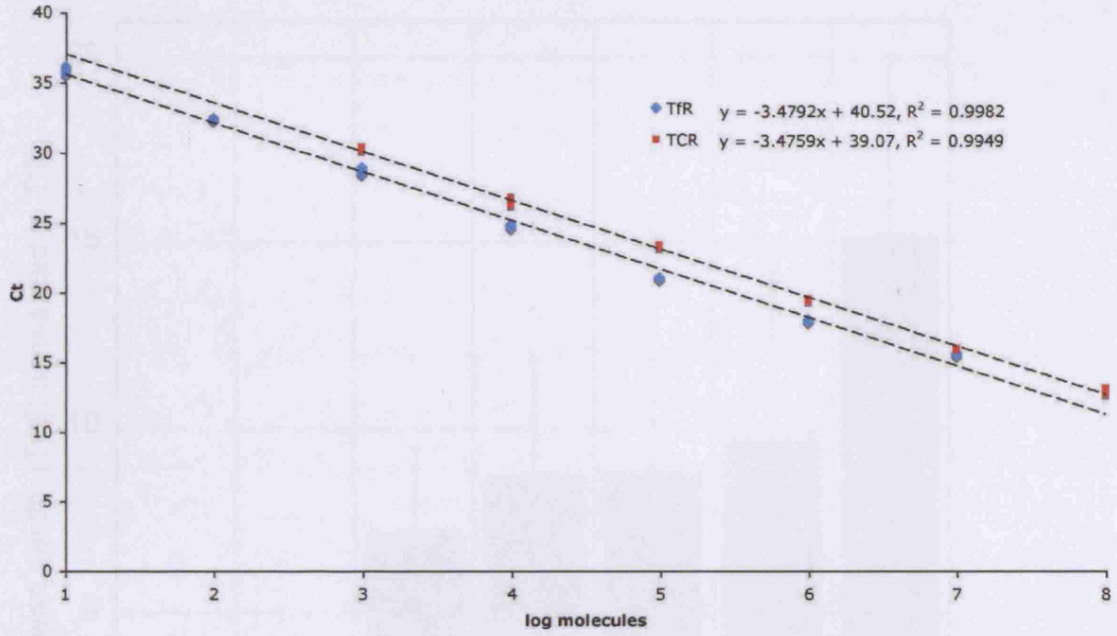


Figure 4-6 Standard curves obtained during qPCR of TCR and TfR dilutions

This graph shows Ct values (threshold cycles: PCR cycle at which a statistically significant increase of the fluorescent signal was first detected) measured by qPCR of standards containing 10^1 to 10^8 molecules of target cDNA.

From these standard curves the efficiency of primer pairs can be calculated as follows.

$$primer\ efficiency = \left[10^{\left(-\frac{1}{slope}\right)} - 1\right] x100$$

Primer efficiencies were similar for amplifications from TCR (93.95 %) and TfR (93.83 %) target cDNA. Quantities of TCR and TfR transcripts obtained from quadruplicate measurements of cDNA from PBMC after stimulation with PHA for different time periods were measured (in repeated experiments). The ratio of mean TCR transcripts in cells normalised to mean transcripts of the constitutive gene TfR (calculated from quadruplicate measurements) can be seen in Figure 4-7.

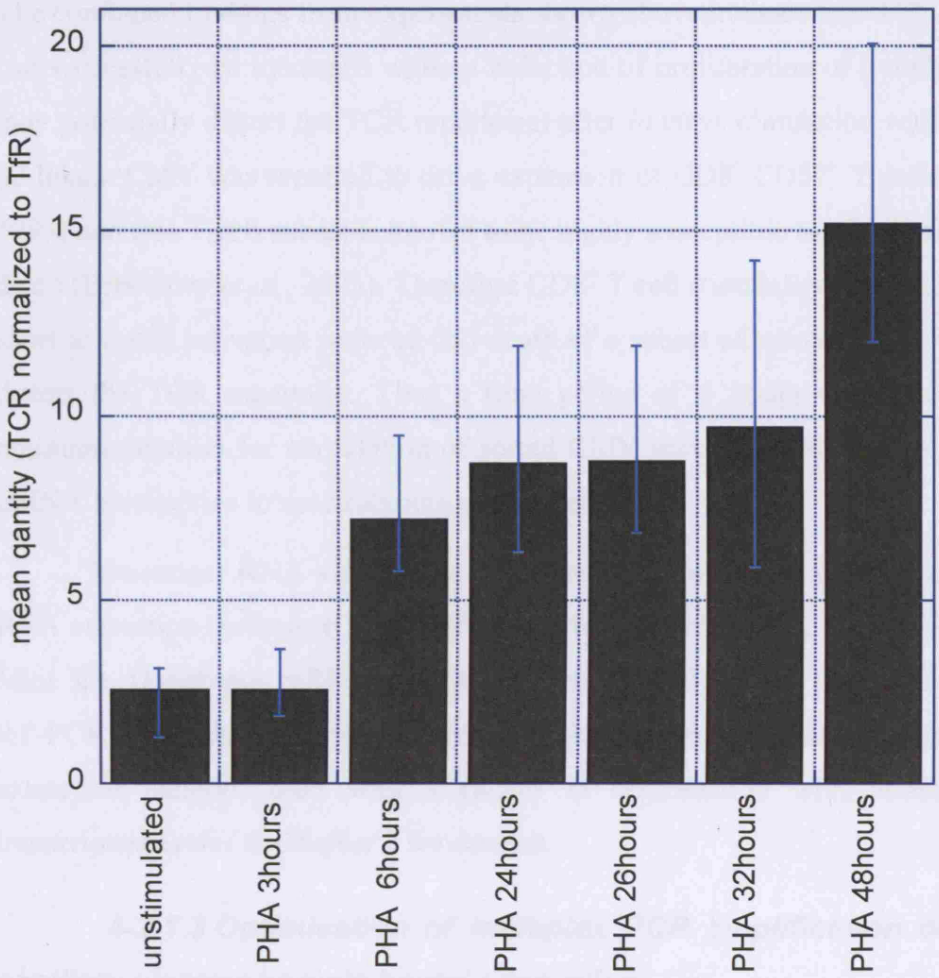


Figure 4-7 Quantification of TCR transcript in lymphocytes after stimulation with PHA

The ratio of the mean quantities of TCR and TfR transcripts (shown with deviations from quadruplicate measurements) is shown for cells stimulated with PHA for the time periods shown on the x-axis.

Values obtained by qPCR were not normalised as transcripts per fixed amount of RNA or cDNA (compare section 2-8.9.1) but are shown as mean transcripts obtained from a fixed number of cells, which were stimulated with PHA. It was decided to set up the experiment in a way that reflects circumstances which would occur in final experiments (using FACS sorted CMV specific T cells after a short stimulation with PHA) to establish whether the balance of cells dying in culture *versus* activation of cells may result in a net increase of TCR mRNA yields from cells. The findings demonstrate that TCR mRNA levels were significantly increased in cells after stimulation with PHA for 6 hours. Levels remained relatively stable during longer periods of stimulation and increased again after a stimulation period of 48 hours in parallel with the start of cell proliferation (compare Figure 4-5).

The combined findings from experiments shown above indicate that TCR mRNA levels can successfully be increased without induction of proliferation of lymphocytes (which may potentially distort the TCR repertoire) after *in vitro* stimulation with PHA for 6 to 32 hours. CMV was reported to drive expansion of $CD8^+$ $CD57^+$ T cells (Wang *et al.*, 1993) and this T cell subset is known to be highly susceptible to activation-induced cell death (Brenchley *et al.*, 2003). Therefore $CD8^+$ T cell stimulation with PHA is best kept short to avoid activation induced cell death of a subset of memory T cells, which may distort the TCR repertoire. Thus a time period of 6 hours was established as the optimum duration for stimulation of sorted CMV specific $CD8^+$ T cells to boost TCR mRNA levels prior to spectratyping experiments.

Messenger RNA yields were additionally optimised by comparison of different RNA extraction methods (Qiagen RNeasy Mini Kit, robot based Qiagen EZ1 RNA Cell Mini Kit, Dynabeads mRNA DIRECT Micro Kit and Qiagen RNeasy Micro Kit) and RT-PCR methods. Best results were obtained from the latter column-based RNA extraction method, used with a carrier in combination with Sensiscript reverse transcriptase (refer to Chapter 2 for details).

4-2.1.3 Optimisation of multiplex PCR amplification of TCR Vß & capillary electrophoresis based separation

The introductory chapter (section 1-8.1) outlined molecular mechanisms leading to diversity of T cell receptors during maturation of $CD8^+$ T cells. During PCR amplifications spanning the CDR3 region between Vß and Cß rearranged gene segments, a single primer can anneal to both the Cß1 and the Cß2 gene segments that can make up the TCR. It is, however, not possible to design a single primer that can anneal to all the Vß gene segments that exist. TCR ß variable gene segments are classified into Vß families based on nucleotide sequence similarity (Rowen *et al.*, 1996, Wei *et al.*, 1994). New Vß gene segments are still being discovered and new families being defined. Spectratyping requires multiple PCR reactions, each including a Cß specific and a Vß family specific primer (refer to section 1-8.2 for details).

Singleplex PCR covering all 24 Vß families which were defined in 1991 (Ferradini *et al.*, 1991) was used initially. This required 26 reactions since separate primers were required to cover the Vß5.1 and Vß5.2 subfamilies and the Vß13.1 and Vß13.2 subfamilies. Figure 4-8 demonstrates separation of PCR products from all of those 26 reactions using singleplex Vß PCR.

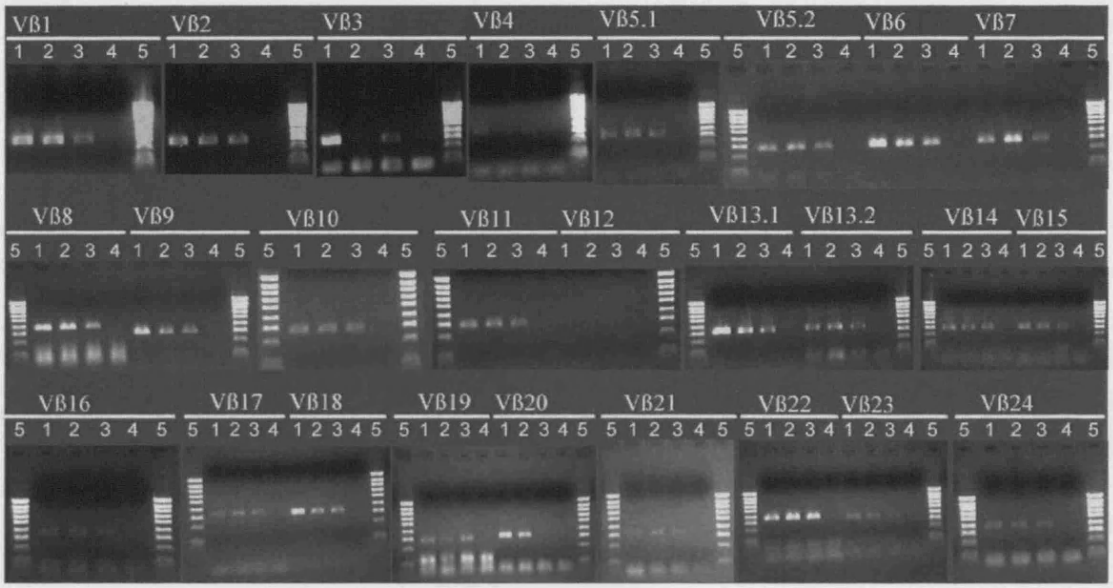


Figure 4-8 Detection of TCR Vß singleplex PCR products by agarose gel electrophoresis

1% agarose gel electrophoresis of PCR products amplified with primers specific for Vß families indicated above the gels. Lane 1, 2 and 3: PCR was performed on cDNA generated from $2x10^6$ PBMC from healthy volunteers 15, 16 and 2 respectively. Lane 4: PCR was performed on control samples using H_2O as starting material for cDNA generation. Lane 5: DNA molecular weight marker (Hyperladder IV, Bioline, compare Figure 4-10 for sizes of standards)

Amplification was successful for all Vß families except Vß12, for which amplification was eventually resolved, as addressed later. PCR products from all other TCR Vß families were successfully detected. PCR products of Vß4 and 16 that were barely visible on the agarose gels shown in Figure 4-8 were subsequently detected by more sensitive methods. After gel electrophoresis was used to confirm successful PCR amplification of TCR Vß templates, PCR products were separated using large polyacrylamide gels, which have a more uniform pore size than agarose gels and therefore enable highly sensitive detection and high resolution separation of DNA fragments as required for spectratyping. This high-resolution separation was performed using 6 % denaturing polyacrylamide gels on an automated DNA sequencer with fragment analysis option. A representative result obtained with this method is shown in Figure 4-9.

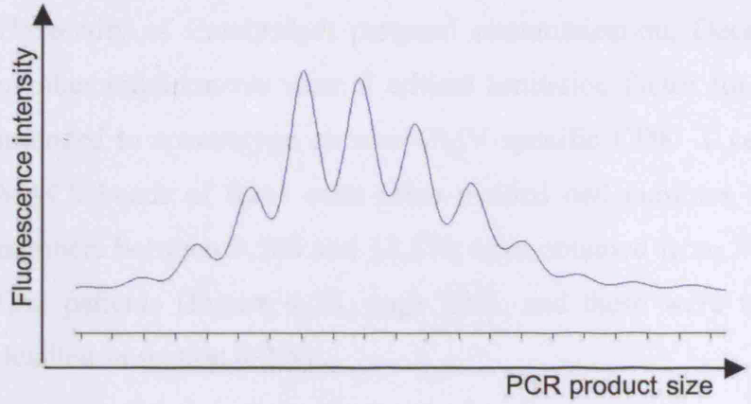


Figure 4-9 Detection of TCR Vß singleplex PCR products by polyacrylamide gel electrophoresis on a DNA sequencer

Frequency histogram of a typical Vß PCR profile from cDNA generated from $5x10^6$ PBMC from a healthy volunteer.

During initial experiments using RNeasy mini kit (Qiagen) without PHA stimulation, followed by singleplex PCR, it was only possible to obtain the minimum amount of RNA necessary to perform spectratyping when using at least $2x10^5$ PBMC. This is illustrated in Figure 4-10.

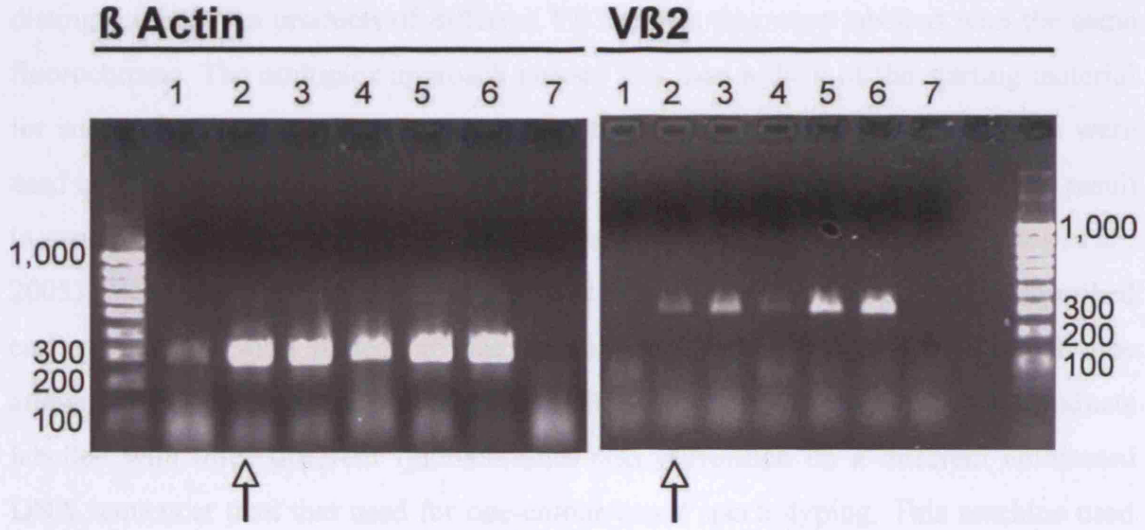


Figure 4-10 Requirement of 200,000 PBMC for spectratyping prior to optimisation

The 1 % agarose gel shows separation of ß-actin (left) *versus* Vß2 (right) PCR products of cDNA generated from 100,000 (lane 1), 200,000 (lane 2), 250,000 (lane 3), 500,000 (lane 4), $1x10^6$ (lane 5) and $2x10^6$ (lane 6) PBMC from a healthy volunteer along with an H_2O control used for cDNA generation (lane 7). The 8th lane in the right gel is empty; lanes on the far left and right represent Hyperladder IV (Bioline) with relevant sizes indicated beside the gel. The arrow indicates minimum cell number requirement for visible PCR amplification

The finding is consistent with observations by other groups (Elizabeth K. Day, University of Cambridge, personal communication, December 2004). Minimum cell number requirements were a critical limitation factor for experiments because it was intended to spectratype *ex vivo* CMV specific $CD8^+$ T cells, and initial sorting using MACS beads of these cells often yielded cell numbers not exceeding 3,000 (higher numbers between 3,500 and 13,870 were obtained from MACS bead sorted cells from four patients (Figure 4-20, page 295), and these were used for further experiments detailed in section 4-2.5).

To improve the lower limit of cell numbers required for spectratyping, a new method was adopted. Instead of conventional use of one labelled Cß primer with one of 26 unlabelled Vß primers in 26 single PCR reactions, an unlabelled Cß primer was used along with Vß primers that were labelled with one of three different fluorochromes and multiplexed into 7 reactions containing the Cß and 2 to 5 Vß primers per reaction (refer to Table 2-7 on page 165 for details). Only 24 Vß primers were used because Vß families 10 and 19 were reported to be pseudogenes (Currier *et al.*, 1996). Multiplexing was based on compatibility of primers (such as similar melting temperatures) and PCR product sizes that needed to be sufficiently distinct from each other in order to distinguish between products of different Vß families that were labelled with the same fluorochrome. The multiplex approach needed less than a third of the starting material for successful TCR spectratyping compared to 26 singleplex PCR reactions that were used initially. In addition multiplex PCR with Vß primer labelling was reported to result in superior resolution when using small amounts of starting material (Fernandes *et al.*, 2005). New primer sequences also resolved Vß12 amplification problems described earlier. The use of a molecular size standard labelled with a fourth fluorochrome allowed for accurate sizing of labelled PCR products. Separation of PCR products labelled with three different fluorochromes was performed on a different automated DNA sequencer than that used for one-colour-based spectratyping. This machine used denaturing capillary electrophoresis with a linear polyacrylamide separation polymer, which resulted in a higher turnover and greater reproducibility of spectratyping.

Figure 4-11 demonstrates separation of TCR Vß PCR products by capillary electrophoresis visualised by Gene Profiler software. Fragments from FAM, JOE or TAMRA labelled PCR products and an ET-ROX labelled size standard were run in the same capillary, resulting in separation of fragments according to size (along the x-axis), which can be detected as fluorescent signals according to their abundance in different

spectral channels (shown on the y-axis). The original software output (after zooming onto part of the y-axis) from separation of a FAM labelled control PCR product is shown in Figure 4-11.

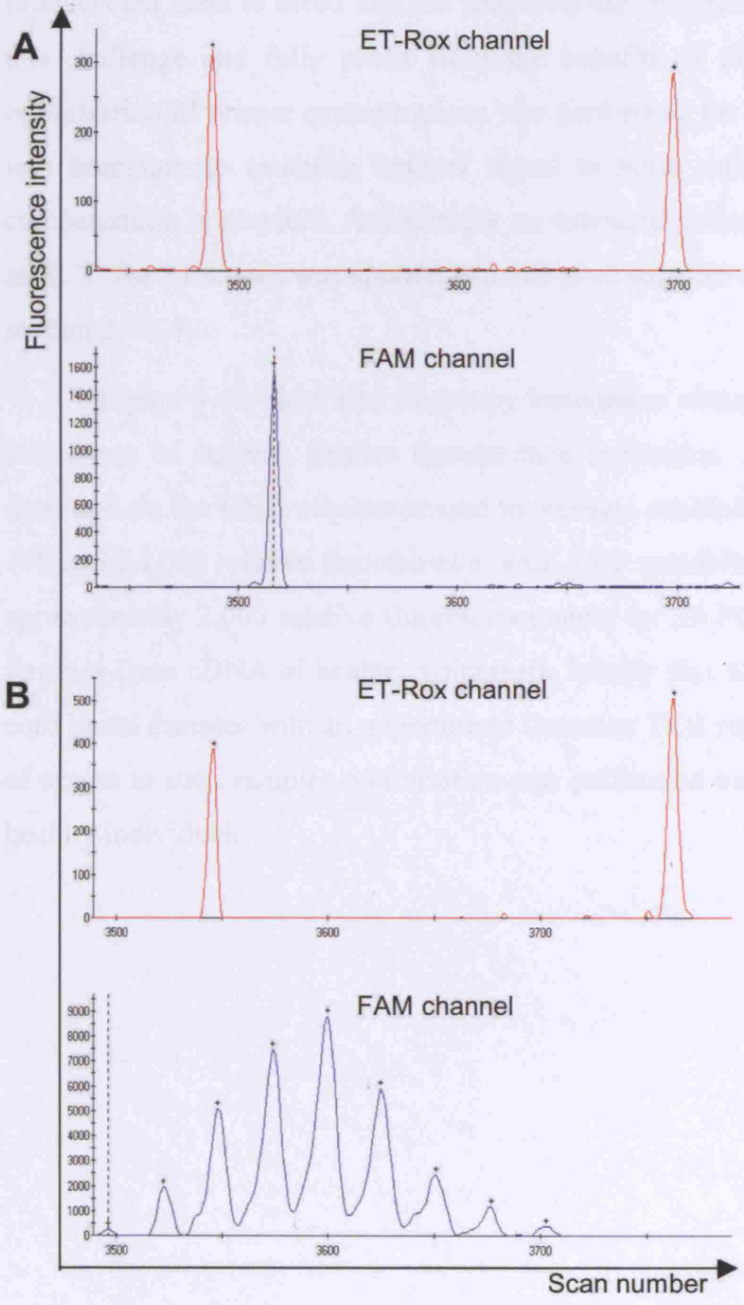


Figure 4-11 Separation of TCR Vß multiplex PCR control products along with a molecular size marker by capillary electrophoresis

Frequency histograms demonstrate the ET-ROX molecular size marker in upper lanes (red) and FAM labelled PCR products in lower lanes (blue). CDR3ß lengths from TCRs of unsorted PBMC from a healthy control follow a classical Gaussian distribution (part B: Vß6 PCR was performed on cDNA generated from $2x10^6$ PBMC) whereas the TCR of (174xCEM) T2 cells demonstrate a uniform CDR3ß length because this human T-B hybrid lymphoblastoid cell line expresses a single T cell receptor (part A: Vß9 PCR was performed on cDNA generated from 3,000 T2 cells).

Initial TCR Vß amplification using similar concentrations of Vß specific primers in PCR reactions revealed that some primers amplified products at a significantly higher level compared to others. The high intensities of the signal from these highly amplified products led them to bleed into the neighbouring channels of the detector. To overcome this challenge and fully profit from the benefits of the new technology, extensive optimisation of primer concentrations was performed for each multiplex Vß PCR. This was necessary to establish optimal signal to noise ratios in which optimal spectral compensation is possible. Additionally an extended period of the primer extension step at 72 °C for 5 minutes was applied and this gave superior amplification results (compare section 2-12.4).

Figure 4-12 illustrates frequency histograms obtained after optimisation of PCR conditions to achieve similar fluorescence intensities. The reliable range of signal detection on the DNA sequencer used to separate multiplex PCR products lies between 100 and 64,000 relative fluorescence units. PCR conditions were optimised to achieve approximately 2,000 relative fluorescence units for all PCR amplifications of TCR Vß families from cDNA of healthy volunteers. Ideally this would have been performed on cord blood samples with an unperturbed Gaussian TCR repertoire. However, due to lack of access to such samples optimisation was performed on pooled cDNA from different healthy individuals.

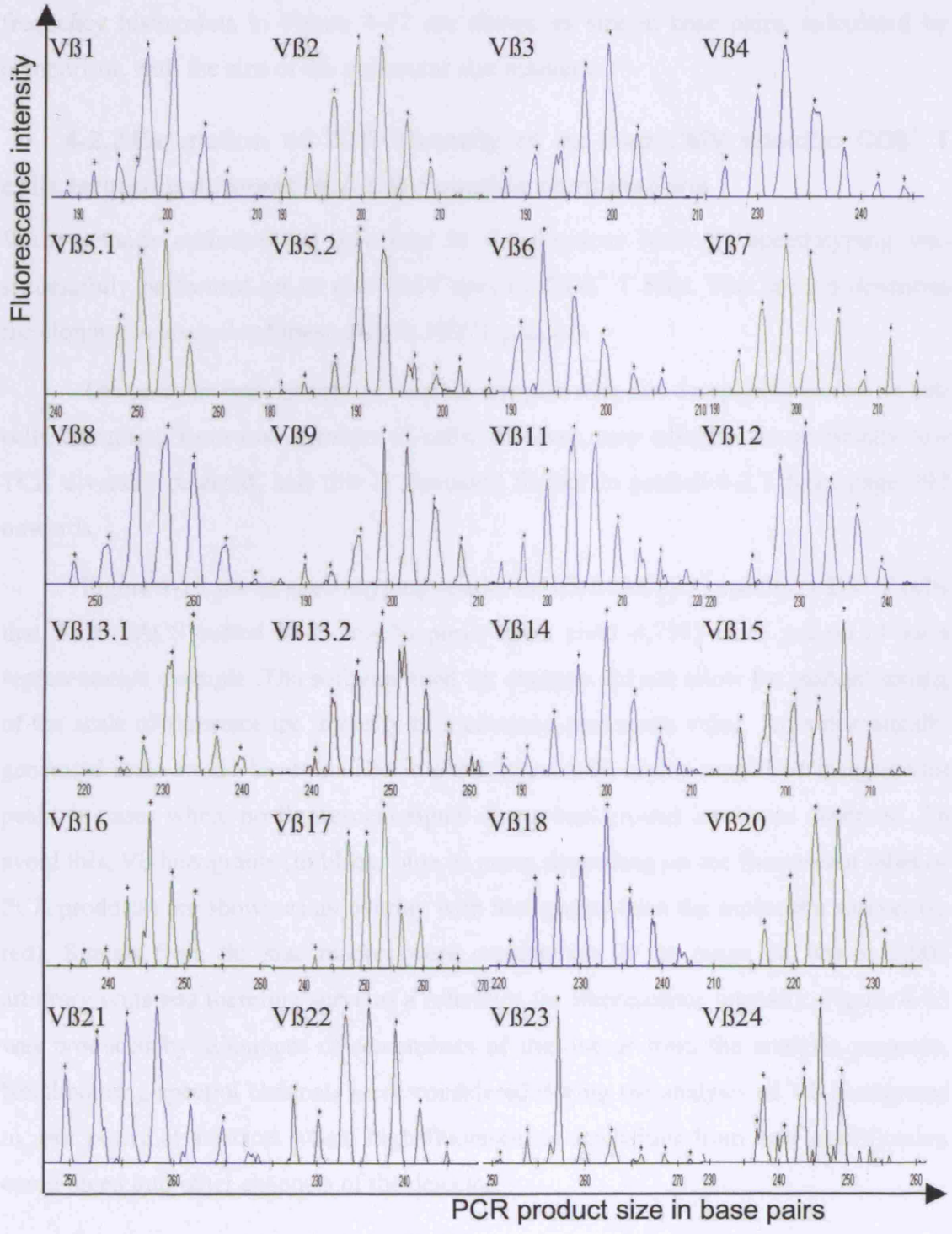


Figure 4-12 Optimised separation and sizing of TCR Vß multiplex PCR products by capillary electrophoresis

Frequency histograms of TCR Vß families derived from unsorted PBMC from healthy volunteers. Primer concentrations were optimised to achieve similar maximum fluorescence intensity (y-axis) of CDR3 spanning TCR Vß PCR products of different sizes (x-axis) from all 24 TCR Vß families (assigned above each histogram).

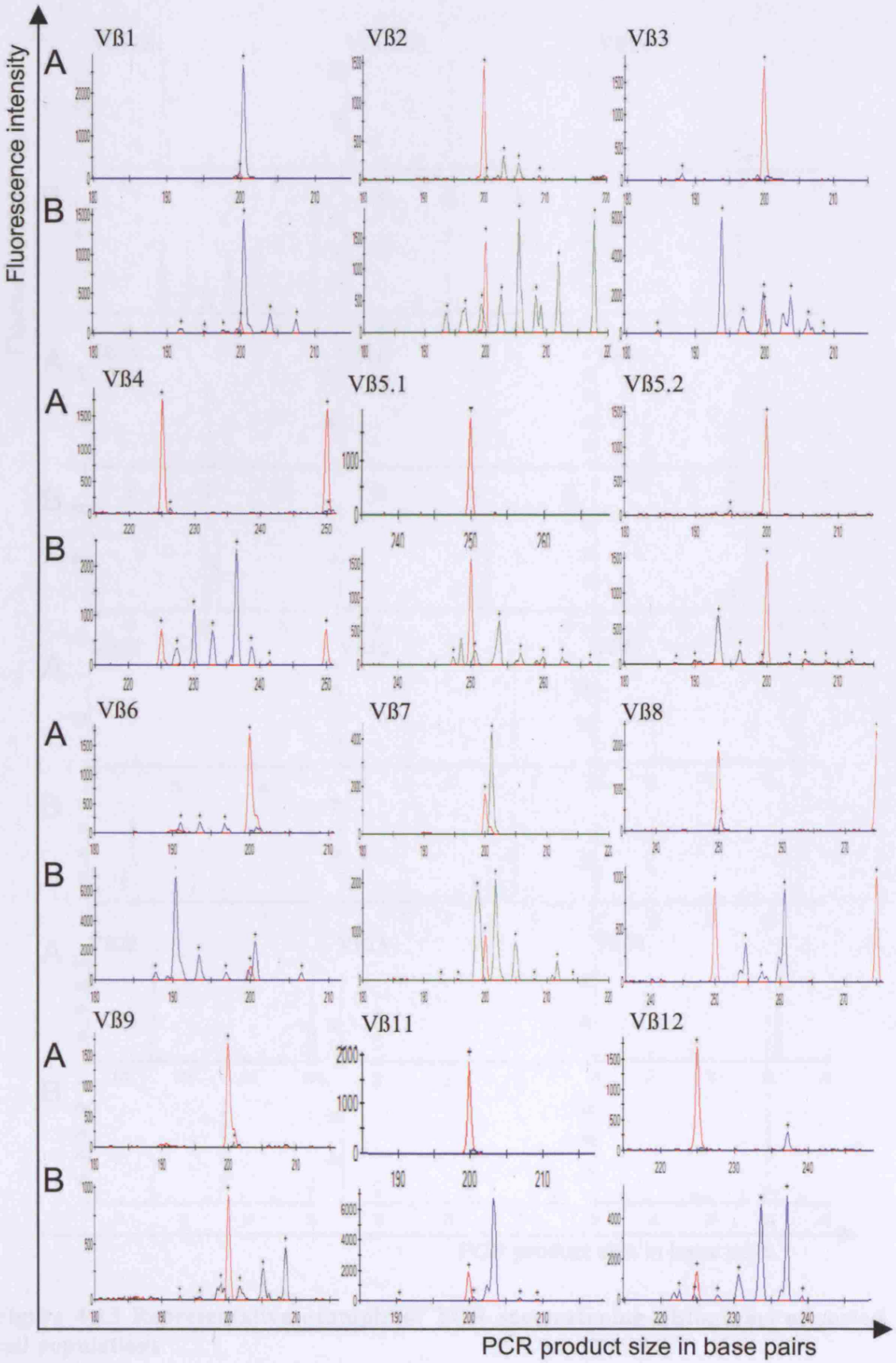
In contrast to original output data as shown in Figure 4-11, the units of the x-axis of frequency histograms in Figure 4-12 are shown as size in base pairs, calculated by comparison with the size of the molecular size markers.

4-2.2 Estimation of TCR diversity of *ex vivo* CMV specific $CD8^+$ T cells targeting different HLA/CMV peptide combinations

With methods optimised as described in the previous sections, spectratyping was successfully performed on *ex vivo* CMV specific $CD8^+$ T cells. This section describes the clonotypic analysis of these cells in HSCT patients.

The analysis was successful on most samples with one comprising as few as 200 cells. Sampling from low numbers of cells, however, may result in an artificially low TCR diversity detected, and this is discussed further in section 4-2.3 from page 292 onwards.

Figure 4-13 shows spectratyping results for HLA-A1/pp50 specific $CD8^+$ T cells that were FACS sorted with 96.4 % purity (cell yield 4,738) from patient 14 as a representative example. The software used for analysis did not allow for manual setting of the scale of fluorescence intensity to a common maximum value. An automatically generated scale would have resulted in illustrations with highly amplified insignificant peaks in cases where no fluorescent signal above background level was observed. To avoid this, Vß histograms (in black, blue or green depending on the fluorescent label of PCR products) are shown as an overlay with histograms from the molecular marker (in red). Signals from the size marker were consistently in the range of 500 to 2,000 arbitrary units and therefore serve as a reference for fluorescence intensity. Figure 4-13 was produced by alignment of screenshots of the output from the analysis program. Neighbouring spectral channels were considered during the analysis of Vß histograms to spot potential artefacts where high fluorescence intensities from one amplification could bleed into other channels of the detector.


Fluorescence intensity
Vß1
Vß2
Vß3
A
B
Vß4
Vß5.1
Vß5.2
Vß6
Vß7
Vß8
Vß9
Vß11
Vß12
PCR product size in base pairs

Continued

Continued

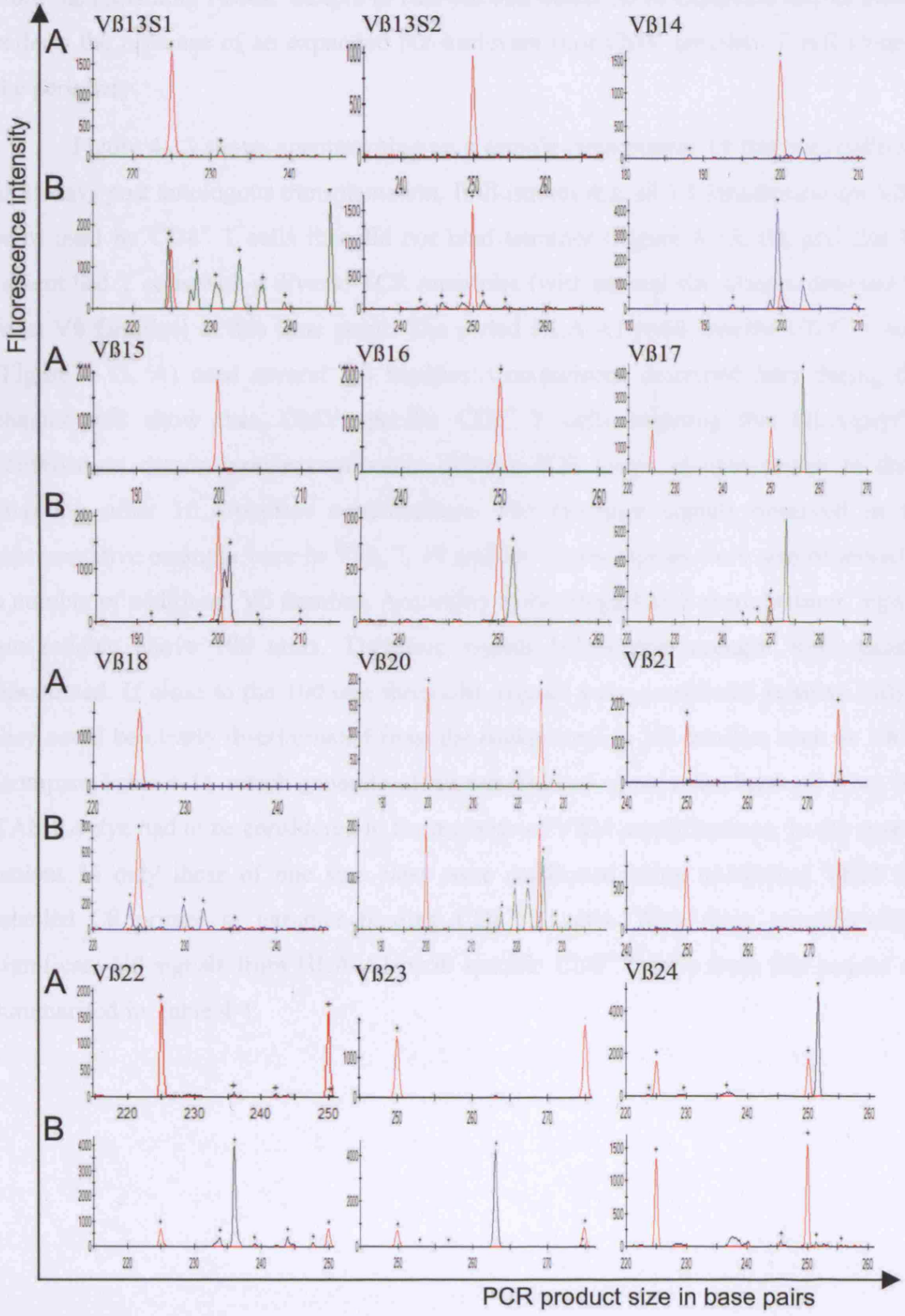


Figure 4-13 Representative example of TCR spectratyping histograms of sorted cell populations

Frequency histograms of TCR Vß families derived from cDNA from patient 14 (black, blue or green curves) in overlay with peaks derived from the molecular size standard (red curve). A: Sorted tetramer binding cell fraction; B: Sorted cell fraction that did not bind tetramer.

Spectratypes of tetramer sorted cells were analysed in comparison with spectratypes from the remaining PBMC sample to rule out that detection of expanded clones merely reflects the presence of an expanded but irrelevant (not CMV specific) T cell clone in the periphery.

Figure 4-13 shows spectratyping on a sample from patient 14 that was collected at 55 days post autologous transplantation. It illustrates that all Vß families except Vß21 were used by $CD8^+$ T cells that did not bind tetramer (Figure 4-13, B), and that the patient had T cells with a diverse TCR repertoire (with several size classes detected for most Vß families) at this time point. The sorted HLA-A1/pp50 specific $CD8^+$ T cells (Figure 4-13, A) used several Vß families. Comparisons described later during this chapter will show that, CMV specific $CD8^+$ T cells targeting this HLA/peptide combination demonstrate exceptionally diverse TCR usage in comparison to those targeting other HLA/peptide combinations. The strongest signals observed in the representative example were in Vß1, 7, 17 and 24. Lower signals were also observed in a number of additional Vß families. According to the MegaBACE manufacturer, signals are reliable above 100 units. Therefore signals below this strength were usually discounted. If close to the 100 unit threshold, signals were considered positive only if they could be clearly discriminated from the background in Vß families such as Vß 22 (compare Table 4-1), which generally demonstrated low signals. Background from free TAMRA dye had to be considered in the analysis of Vß24 amplifications. In the case of patient 14 only those of one size class were confirmed using unlabelled Vß24 and labelled Cß primer in tetramer binding $CD8^+$ T cells. With these considerations, significant Vß signals from HLA-A1/pp50 specific $CD8^+$ T cells from this patient are summarised in Table 4-1.

Vß family	Number of CDR3 lengths	Maximum fluorescence intensity detected
1	1	26,965
2	2	304
3	1	101
6	3	180
7	1	4,248
8	1	312
12	1	305
13S1	1	137
14	1	181
17	2	3,960
20	2	125
22	2	85
24	1	4,947

Table 4-1 Representative example of CDR3 size classes detected using spectratyping of CMV specific CD8$^+$ T cells

List of the number of different TCR Vß CDR3 lengths and fluorescence intensity detected in cDNA from tetramer binding cells from patient 14.

As shown for patient 14 above, spectratyping results were analysed for other patients. Findings are summarised in different sections depending on the HLA/CMV peptide specificity of CD8$^+$ T cells. Since HLA-A2/pp65 specific CD8$^+$ T cells might be considered as an archetype for comparison of CMV specific CD8$^+$ T cells targeting other HLA/peptide combinations (compare section 3-2.4), analysis of these cells will be shown first.

4-2.2.1 TCR diversity of HLA-A2/pp65 specific CD8$^+$ T ceils

HLA-A2/pp65 specific CD8$^+$ T cells are the most studied CMV specific CD8$^+$ T cells and as such might be considered as a standard for comparison with CMV specific CD8$^+$ T cell responses targeting other HLA/peptide combinations. To use them as a standard in this study, the TCR diversity of HLA-A2/pp65 specific CD8$^+$ T cells was studied in this section and findings will be compared to the TCR diversity of other cells in section 4-3.

Spectratyping was successfully performed on HLA-A2/pp65 specific FACS sorted CD8$^+$ T cells from 5 HSCT patients. Results from spectratyping histograms are summarised in Figure 4-14.

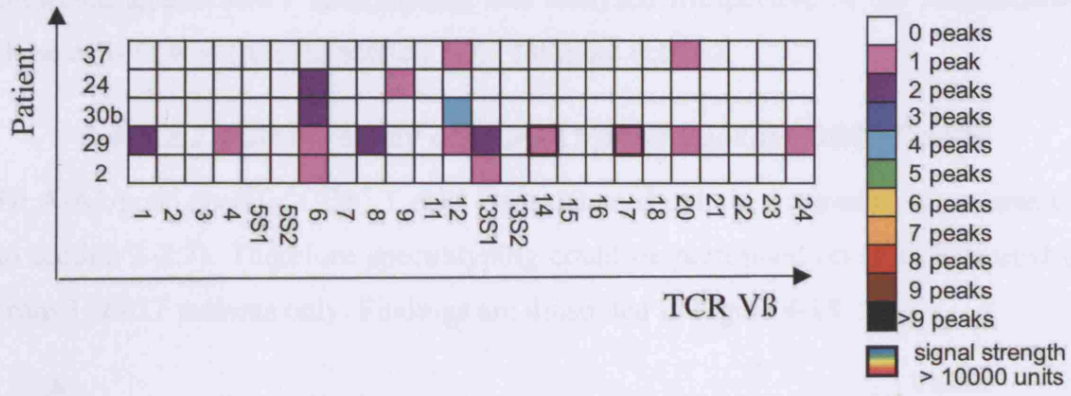


Figure 4-14 CDR3 size classes detected by spectratyping of HLA-A2/pp65 specific CD8$^+$ T cells

CDR3 size classes detected in cDNA from tetramer binding cells from different patients (y-axis) are illustrated by boxes with different colours reflecting the number of different CDR3 lengths (histogram peaks) observed for TCRs in each Vß family (x-axis).

Figure 4-14 demonstrates that HLA-A2/pp65 specific CD8$^+$ T cells obtained from different patients use Vß families 1, 4, 6, 8, 9, 12, 13S1, 14, 17, 20 and 24. The mean number of TCR Vß families used by HLA-A2/pp65 specific CD8$^+$ T cells was 3.6 (n = 5, range 2 - 10), which is similar to observations in CD8$^+$ T cells that target other HLA/peptide combinations (sections 4-2.2.2, 4-2.2.4 and 4-2.2.5). In contrast, CD8$^+$ T cells targeting HLA-A1/pp50 (section 4-2.2.3) used a significantly greater diversity of TCRs.

HLA-A2/pp65 specific CD8$^+$ T cells from 4 out of 5 patients analysed used 2 different Vß families, whereas cells from the remaining patient used 10 Vß families. The number of HLA-A2/pp65 TCR Vß families detected in this study is similar to previous observations, which will be discussed further in section 4-3 (page 312 onwards). Shared CDR3 lengths were observed for the TCRs of HLA-A2/pp65 specific CD8$^+$ T cells for Vß6 in patients 24, 29 and 30b (198 bp), for Vß12 in patients 30b and 37 (234 bp), for Vß12 in patients 29 and 30b (228 bp) and for Vß20 in patients 29 and 37 (226 bp). These CDR3 size classes may reflect public TCR repertoires.

The highest signals detected by Vß spectratyping of HLA-A2/pp65 specific CD8$^+$ T cells were found in patient 29. Paradoxically, this patient demonstrated the most diverse TCR repertoire and the highest level of these cells out of all HSCT patients, but was not protected against CMV reactivation (Figure 3-24, page 233).

The TCR diversity of CD8$^+$ T cells from patient 24 and 29 (both of whom demonstrated high levels of HLA-A2/pp65 specific CD8$^+$ T cells and were not

protected against CMV reactivation) was analysed irrespective of the functionality of these cells *in vivo* (refer to section 3-3.1 for more details).

4-2.2.2 TCR diversity of HLA-A1/pp65 specific CD8$^+$ T cells

HLA-A1/pp65 specific CD8$^+$ T cells were detected only in a minority of patients (refer to section 3-2.3). Therefore spectratyping could be performed on (FACS) sorted cells from 3 HSCT patients only. Findings are illustrated in Figure 4-15.

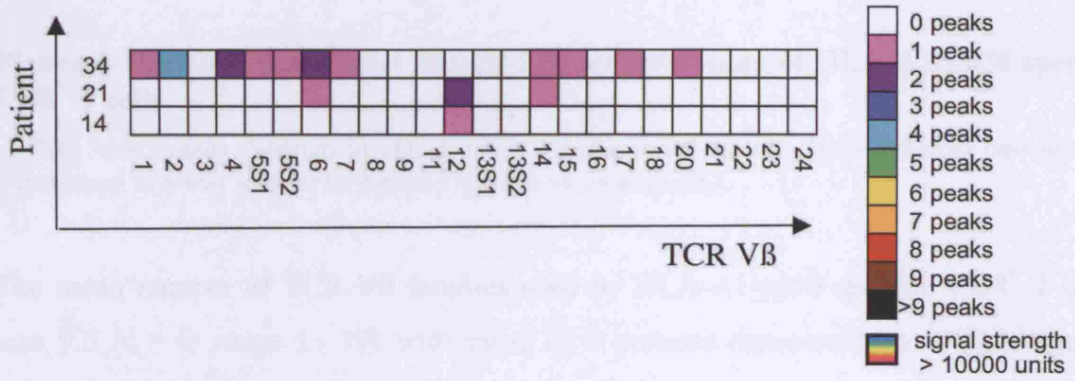


Figure 4-15 CDR3 size classes detected by spectratyping of HLA-A1/pp65 specific CD8$^+$ T cells

CDR3 size classes detected in cDNA from tetramer binding cells from different patients are illustrated in a way similar to that of Figure 4-14 on page 287.

The mean number of TCR Vß families used by HLA-A1/pp65 specific CD8$^+$ T cells was 5 (n = 3, range 1 - 11). A comparison of these cells with CMV specific CD8$^+$ T cells targeting other HLA/CMV peptide combinations will be discussed in section 4-3.2 (page 315). A size class of 234 bp in Vß 12 was shared by patients 14 and 21 and may reflect a public TCR repertoire.

4-2.2.3 TCR diversity of HLA-A1/pp50 specific CD8$^+$ T cells

Spectratyping was successfully performed on HLA-A1/pp50 specific (FACS) sorted CD8$^+$ T cells from four HSCT patients with results shown in Figure 4-16.

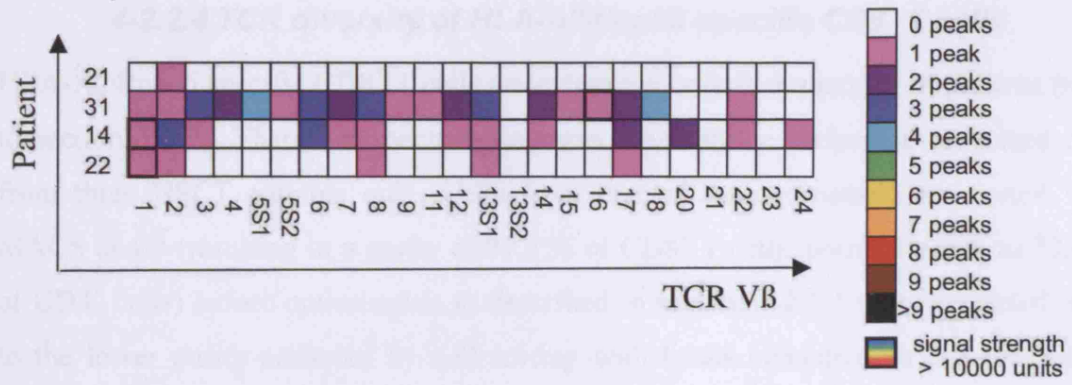


Figure 4-16 CDR3 size classes detected by spectratyping of HLA-A1/pp50 specific CD8$^+$ T cells

CDR3 size classes detected in cDNA from tetramer binding cells from different patients are illustrated in a way similar to that of Figure 4-14 on page 287.

The mean number of TCR Vß families used by HLA-A1/pp50 specific CD8$^+$ T cells was 9.5 (n = 4, range 1 - 19) with three of 4 patients demonstrating TCR usage of exceptionally high diversity, which was significantly greater than that observed for CMV specific CD8$^+$ T cells targeting other HLA/CMV peptide combinations (compare sections 4-2.2.1, 4-2.2.2, 4-2.2.4 and 4-2.2.5). In contrast to other CMV specific CD8$^+$ T cells studied in this project, cells investigated in this section targeted a different CMV protein. All other HLA/CMV peptide combinations involved peptides from the CMV pp65 protein, whereas cells investigated in this section targeted peptides from the CMV pp50 protein.

Similar CDR3 lengths were observed in 5 Vß families. The strongest signals from HLA-A*0101 restricted CMV pp50 (245-253) specific CD8$^+$ T cells were observed for a CDR3 size class of 200 bp from TCR Vß1 in patients 14 and 22. Similar CDR3 lengths of 202 bp were also observed in Vß2 in these two patients. Patients 14 and 31 shared similar CDR3 lengths of 205 bp in Vß2, 251 bp in Vß8 and 231 bp in Vß13S1. Signals from Vß22 were below 100 fluorescence intensity units in tetramer binding cells from patients 14 (Figure 4-13) and 31. Since PCR products were generally of low signal strength and very low background noise was detected in this particular Vß family, these peaks are considered genuine. Accordingly patients 14 and 31 also share a CDR3 length of 242 bp in Vß22. These CDR3 size classes may reflect public TCR repertoires.

4-2.2.4 TCR diversity of HLA-A24/pp65 specific CD8$^+$ T cells

HLA-A24/pp65 specific CD8$^+$ T cells were detected only in a minority of patients (refer to section 3-2.2). Therefore spectratyping was successfully performed on sorted cells from three HSCT patients only. Cells from one of these patients were sorted with MACS beads (resulting in a purity of 97.3 % of CD8$^+$ T cells, corresponding to 70.6 % of CD3$^+$ cells) before optimisation as described in section 4-2.1.1 was completed. Due to the lower purity achieved by cell sorting with beads compared to cell sorting by FACS (compare Figure 4-2 and Figure 4-3 on page 266 and 268 respectively) signals were only considered to be genuine when the strength of fluorescence intensity in tetramer binding cells was similar or greater than the signal strength observed in cells that did not bind tetramer. Findings are illustrated in Figure 4-17.

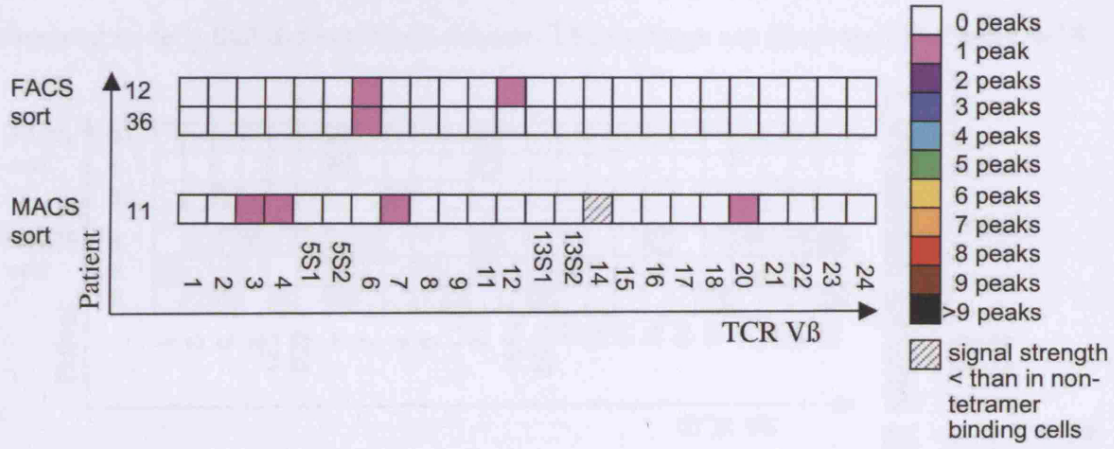


Figure 4-17 CDR3 size classes detected by spectratyping of HLA-A24/pp65 specific CD8$^+$ T cells

CDR3 size classes detected in cDNA from either FACS sorted (upper part) or MACS bead sorted (lower part) tetramer binding cells from different patients (y-axis) are illustrated by boxes with different colours reflecting the number of different CDR3 lengths (histogram peaks) observed for TCRs in each Vß family (x-axis).

Two patients (patient 12 and 36) used the same Vß family. However, similar CDR3 lengths were not observed. The mean number of TCR Vß families used by HLA-A24/pp65 specific CD8$^+$ T cells was 2.3 (n=3, range 1 - 4). CMV specific CD8$^+$ T cells targeting this HLA/CMV peptide combination used a TCR repertoire that was restricted to the same degree as that observed for HLA-B35/pp65 specific CD8$^+$ T cells that will be shown in section 4-2.2.5. HLA-A24/pp65 tetramer sorted cells from patient 11 were further investigated in section 4-2.5.

Considering only those results obtained from samples that were FACS sorted which may therefore be more reliable due to the high purity of cells analysed, the

number of TCR Vß families used by HLA-A24/pp65 specific $CD8^+$ T cells was 1 and 2 respectively (n = 2).

4-2.2.5 TCR diversity of HLA-B35/pp65 specific $CD8^+$ T cells

Spectratyping was successfully performed on HLA-B35/pp65 specific sorted $CD8^+$ T cells from six HSCT patients. Cells from three of those were sorted with MACS beads before optimisation as described in section 4-2.1.1. The purity of sorted cells achieved was only 68.6 % of $CD8^+$ T cells corresponding to 59.1 % of $CD3^+$ T cells in patient 4, 96.3 % of $CD8^+$ T cells corresponding to 56.9 % of $CD3^+$ T cells in patient 5 and 65.3 % of $CD8^+$ T cells corresponding to 56.6 % of $CD3^+$ T cells in patient 9. Signals were therefore only considered to be genuine when the strength of fluorescence intensity in tetramer binding cells was similar or greater than the signal strength observed in cells that did not bind tetramer. The findings are illustrated in Figure 4-18.

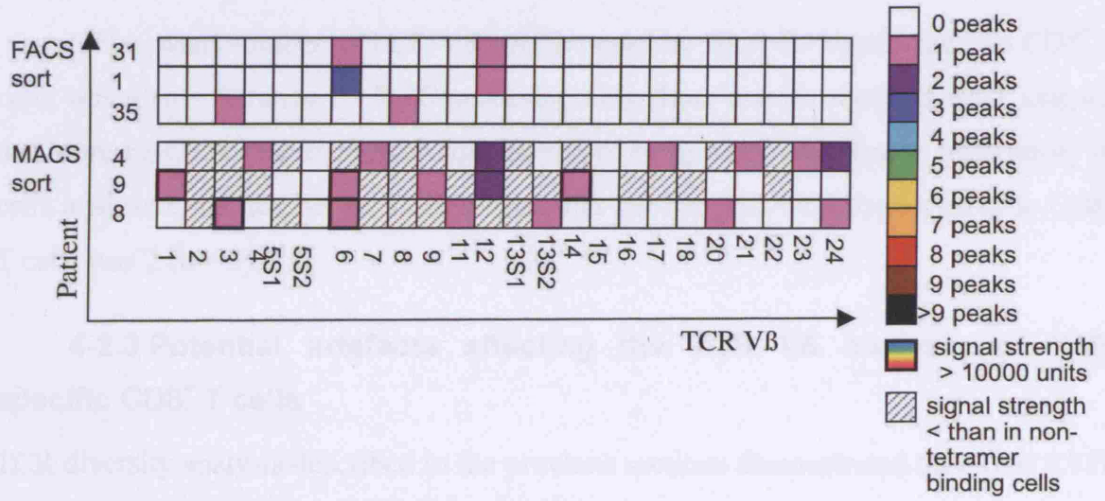


Figure 4-18 CDR3 size classes detected by spectratyping of HLA-B35/pp65 specific $CD8^+$ T cells

CDR3 size classes detected in cDNA from sorted tetramer binding cells from different patients are illustrated in a way similar to that of Figure 4-17 on page 290.

Similar CDR3 lengths were observed in four Vß families.

- In Vß6, a CDR3 size class of 194 bp was observed in patients 1, 8 and 9. However, the signal strength observed in tetramer binding cells was lower than in cells that did not bind tetramer from patients 8 and 9 and therefore this CDR3 length was not considered genuine and is not illustrated for patients 8 and 9 in Figure 4-18. Also within this Vß family, a CDR3 size class of 197 bp was observed in patients 1, 4 and 9. For this CDR3 length, lower signal strength in

tetramer binding cells than in cells that did not bind tetramer was observed in patient 4 (not considered genuine). An additional Vß6 CDR3 size class of 206 bp was observed in patients 4 and 8 but not considered genuine in patient 4.

- In Vß 12, a CDR3 size class of 221 bp was observed in patients 1, 9 and 31 and a CDR3 size class of 227 bp was observed in patients 4, 8 and 9 (the latter not considered genuine in patient 8).
- In Vß3, a CDR3 size class of 200 bp was observed in patients 4, 8, 9 and 35. This was considered genuine in patients 8 and 35 only and will be further discussed in section 4-3.
- In Vß24, a CDR3 size class of 243 bp was observed in patients 4 and 8.

These CDR3 size classes reflect potentially public TCR repertoires and some of them (Vß6 and Vß12) were further investigated in section 4-2.5.

The mean number of TCR Vß families used by HLA-B35/pp65 specific $CD8^+$ T cells was 4 (n = 6, range 2 - 7). Considering only those results obtained from samples that were FACS sorted and which may therefore be more reliable due to high purity of cells analysed, the number of TCR Vß families used by HLA-B35/pp65 specific $CD8^+$ T cells was 2 (n = 3).

4-2.3 Potential artefacts affecting the TCR Vß analysis of CMV specific $CD8^+$ T cells

TCR diversity analysis described in the previous sections demonstrated that most CMV specific $CD8^+$ T cells used Vß families 6 and/or 12. This is summarised in Figure 4-19.

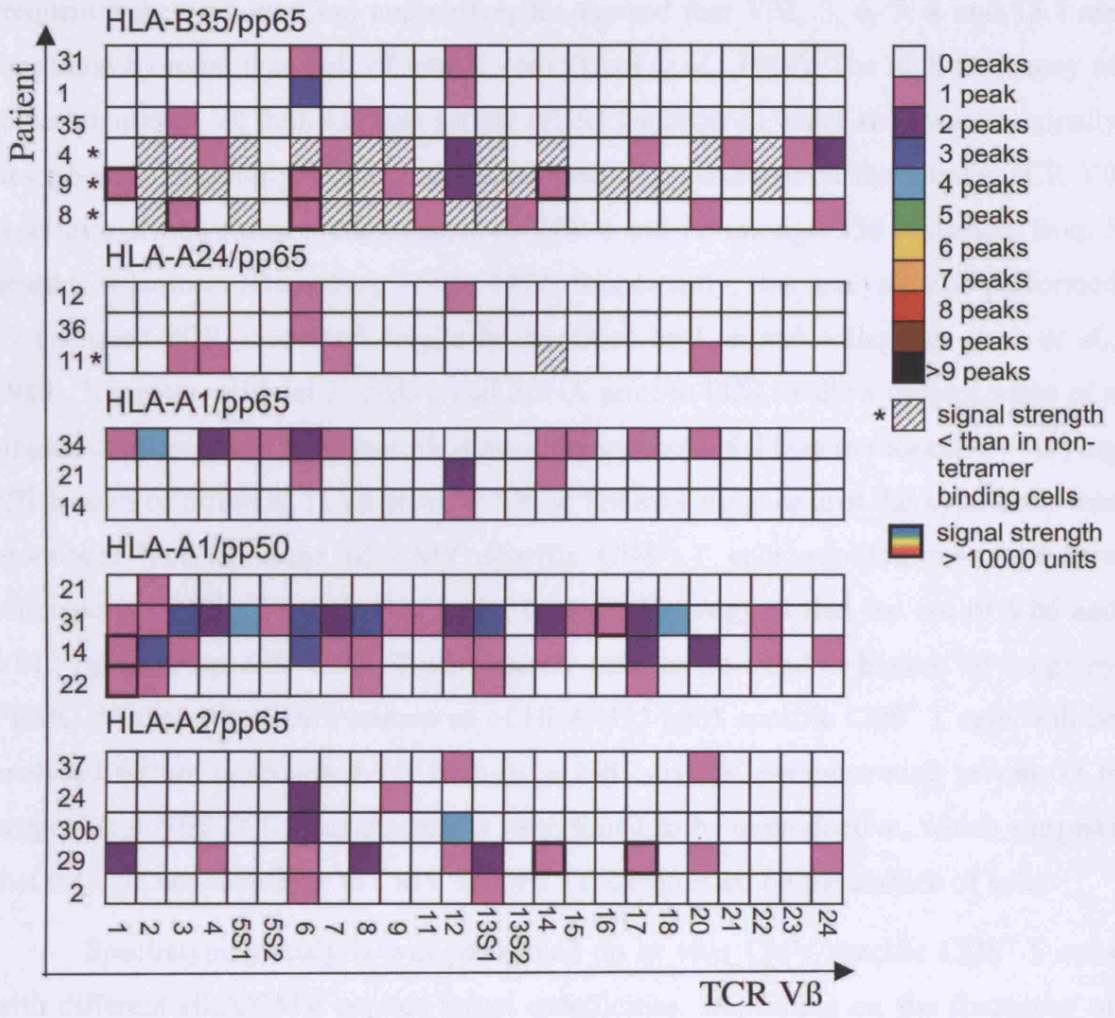


Figure 4-19 CDR3 size classes detected by spectratyping of CMV specific CD8$^+$ T cells targeting different HLA/peptide combinations

CDR3 size classes detected in cDNA from FACS or bead (*) sorted tetramer binding cells from HSCT patients (patient numbers indicated on the left). Different colours reflect the number of different CDR3 lengths (histogram peaks) observed for TCRs in each Vß family.

This finding is surprising. As mentioned in section 1-8.1, CDR1 and CDR2 encoded in the germline V segment genes are thought to interact primarily with the MHC. Therefore, it may be expected that CMV peptides presented by different HLA may be recognised by T cell receptors with different CDR1 and CDR2, and therefore different Vß families.

The common usage of Vß6 and Vß12 in different responses raises the question of whether this finding may have resulted from an artefact introduced by variable efficiencies of primers specific for different Vß families. Another possible explanation for this finding would be differential usage of Vß families by PBMC from the general population. It is known that normal peripheral T cells use some Vß families more

frequently than others. Choi and colleagues showed that Vß2, 3, 6, 7, 8 and 13.1 are expressed by more than half of total T cells (Choi *et al.*, 1989). The high frequency of lower-numbered Vß families may simply reflect the order in which they were originally described. Interestingly, a study aimed at analysing variations in the human TCR Vß repertoire demonstrated predominance of Vß6, 4 and 12 amongst 250 sequences from 5 healthy volunteers (Rosenberg *et al.*, 1992). Importantly, this analysis was performed by anchored PCR, a method originally described by Loh and colleagues (Loh *et al.*, 1989). It creates artificial 3' ends on all cDNA prior to PCR to allow hybridization of a single complementary 3' primer, thus avoiding any potential bias introduced by varying efficiencies of different 3' Vß primers. These findings argue against the hypothesis that prominent Vß6/12 usage of CMV specific $CD8^+$ T cells resulted from different efficiencies of Vß primers in this study. Instead, they suggest that the use of Vß6 and Vß12 by CMV specific $CD8^+$ T cells merely reflects the trend in biased Vß usage by PBMC. Vß6 and 12 CDR3 sequences of HLA-B35/pp65 specific $CD8^+$ T cells will be analysed further in section 4-2.5 from page 298 onwards, demonstrating private TCR usage. Most Vß6 TCR rearrangements were found to be unproductive, which suggests that they did not contribute to CMV specific TCR expressed on the surface of cells.

Spectratyping analysis was performed on *ex vivo* CMV specific $CD8^+$ T cells with different HLA/CMV peptide target specificities. Depending on the frequency of these cells in PBMC, cell numbers obtained by tetramer guided sorting differed substantially between patients. Clonotypic analysis was performed on a range of 200 to nearly 74,000 FACS sorted cells. This raises the possibility of a sampling artefact: Samples with low cell numbers may have artificially low TCR diversity. This may be due to lower amounts of cDNA generated from such samples, which, in turn, may promote weaker PCR amplification signals and therefore detection of less CDR3 size classes. Numbers of TCR Vß families detected in tetramer sorted cells were therefore plotted against the number of cells used for this analysis in an attempt to test if both factors correlate, which may suggest a sampling artefact (Figure 4-20).

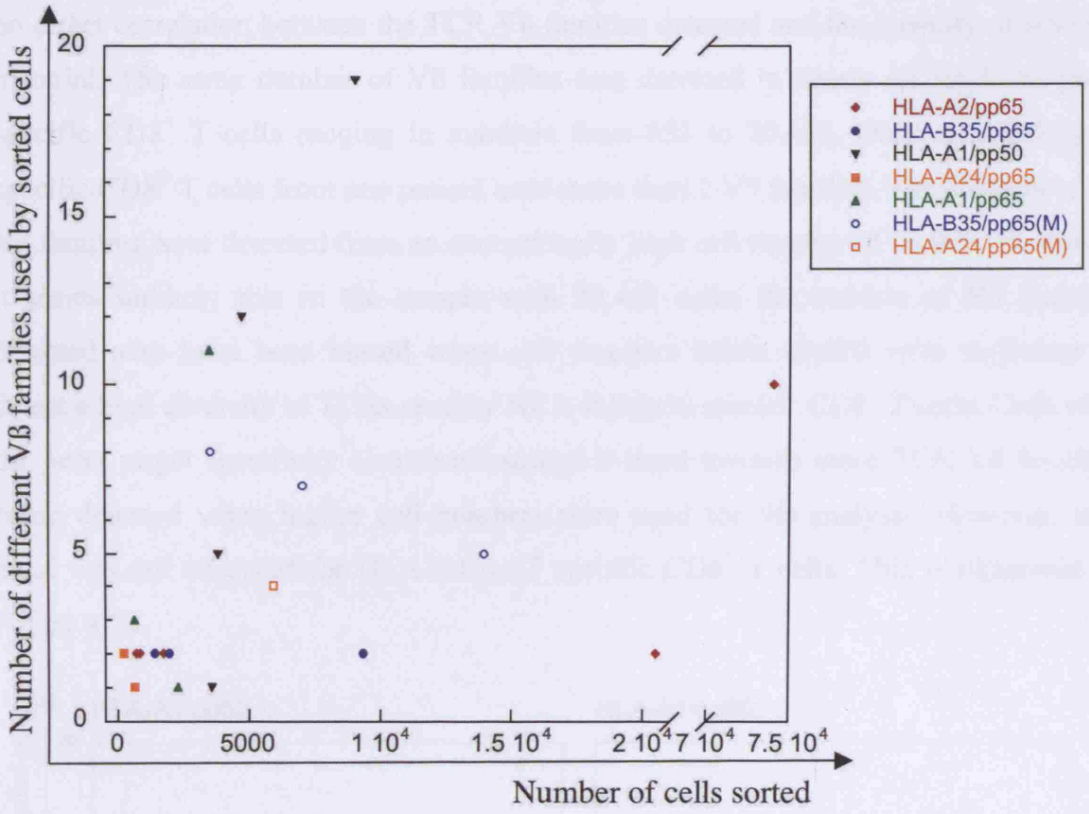


Figure 4-20 Comparison of TCR diversity of CMV specific CD8$^+$ T cells with different cell numbers used for analysis

This scatter graph shows a plot of numbers of sorted cells used for analysis *versus* numbers of Vß families that were detected by spectratyping of these sorted cells. Differently shaped symbols distinguish data points according to the target specificity of cells (M: MACS bead sorted cells).

Findings illustrated in Figure 4-20 demonstrate that there was no overall correlation between numbers of Vß families detected and numbers of cells used for analysis. CMV specific CD8$^+$ T cells that used the highest numbers (12 and 19) of TCR Vß families were not derived from samples with the highest cell numbers. Instead they were derived from cells sorted with HLA-A1/pp50 tetramer, whereas cells sorted with other tetramers used ≤11 TCR Vß families. This suggests that usage of different numbers of Vß gene families was dependent on HLA/peptide specificity of cells.

For HLA-B35/pp65 and HLA-A24/pp65 specific CD8$^+$ T cells, MACS bead sorting resulted in detection of more Vß families than FACS sorting which is likely to have resulted from the lower purity of cells obtained with the bead method (compare section 4-2.1.1, page 265). Bead or FACS sorted HLA-B35/pp65 or HLA-A24/pp65 specific TCR Vß families detected did not correlate with the quantity of starting

material. Other target specificities demonstrate a trend towards a positive correlation but no direct correlation between the TCR Vß families detected and the quantity of starting material. The same number of Vß families was detected in nearly all HLA-A2/pp65 specific $CD8^+$ T cells ranging in numbers from 651 to 20,468. Only HLA-A2/pp65 specific $CD8^+$ T cells from one patient used more than 2 Vß families. For this patient 10 Vß families were detected from an exceptionally high cell number of 73,977. However, it seems unlikely that in the sample with 20,468 cells, the number of Vß families detected may have been biased when cell numbers below 10,000 were sufficient to detect a high diversity of TCRs used by HLA-A1/pp50 specific $CD8^+$ T cells. Cells with the latter target specificity also demonstrated a trend towards more TCR Vß families being detected when higher cell numbers were used for the analysis. However, this trend was not observed for HLA-A1/pp65 specific $CD8^+$ T cells. This is illustrated in Figure 4-21.

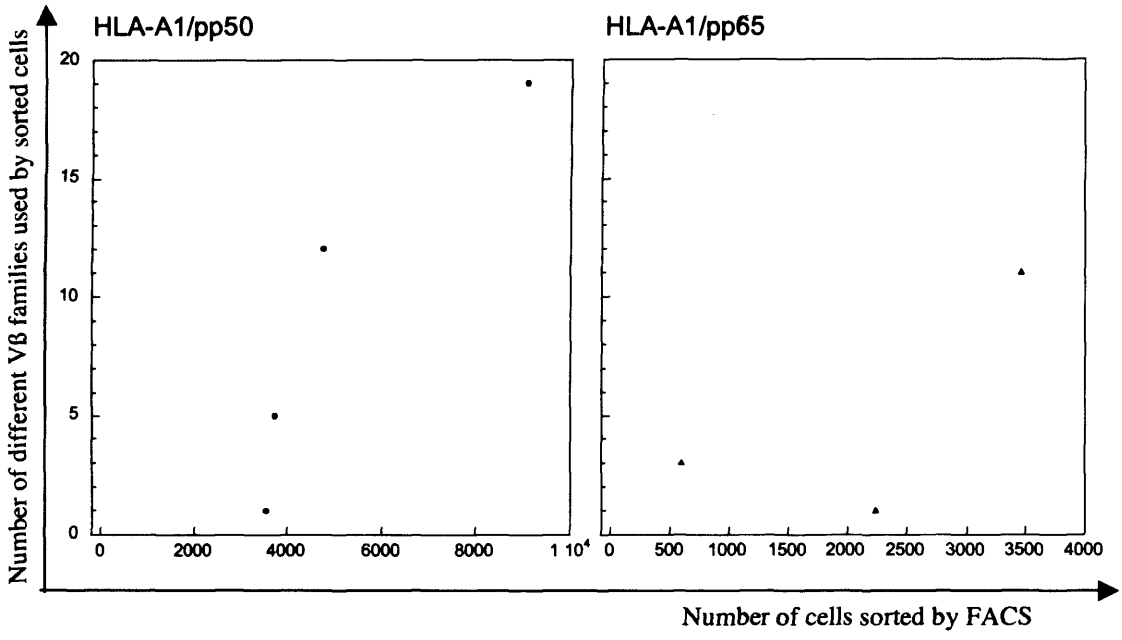


Figure 4-21 Comparison of TCR diversity of HLA-A1 restricted CMV specific $CD8^+$ T cells with different cell numbers used for analysis

Scatter graphs show the numbers of sorted cells used for analysis *versus* numbers of Vß families that were detected by spectratyping of sorted cells that bound HLA-A1/pp50 tetramer (left) or HLA-A1/pp65 tetramer (right).

With a trend towards positive correlation observed for cells with some target specificities but not others, from a relatively small data set, it remains undetermined if this observation was merely a result of chance. Given these observations, a biased detection of different numbers of TCR Vß families of CMV specific $CD8^+$ T cells by

analysis of different numbers of sorted cells cannot be ruled out. It may be informative to use cells with detectable CDR3 size classes within several Vß families for an additional experiment in future studies. These cells could be split into two samples each, one representing a high cell number, a second one representing a low cell number, for use in spectratyping analysis in parallel. This may prove directly whether the TCR repertoire observed in the sample with a high cell number may be skewed in the sample with a lower cell number. Provided that enough material is available, a test series of samples with different cell numbers may be analysed to establish the threshold of cell numbers at which skewing of the TCR diversity can be observed. Due to restricted material and resources it was not feasible to perform this analysis during this project.

Consistency of results for cDNA derived from >70,000 T cells but not from cDNA derived from 35,000 T cells was reported by Manfras and colleagues (Manfras *et al.*, 1997). Findings from that study cannot be directly applied to the different settings used for spectratyping in this study but they suggest that low cell numbers can give rise to a sampling artefact.

4-2.4 Quantification of the constitutive gene TfR in samples that failed to produce TCR Vß PCR products

TCR diversity of *ex vivo* CMV specific $CD8^+$ T cells was estimated in the previous sections by tetramer guided sorting of CMV specific T cells followed by PCR amplification of the CDR3 spanning TCR Vß region from their cDNA and separation of fluorescently labelled PCR products of different lengths by capillary electrophoresis. A signal from PCR products was not obtained from all samples prepared. At least one sample per batch stained with a specific HLA/CMV tetramer (with a total of ten samples) did not result in detection of a Vß amplification signal. The success rate was 68 %. This was not dependent on the numbers of sorted cells or due to failing of a particular PCR reaction but was confirmed to be the result of insufficient cDNA quantity or quality obtained from these samples. This was shown by testing of cDNA for amplification of a constitutive gene by quantitative PCR as described in section 4-2.1.2 (page 268). Results are illustrated in Figure 4-22.

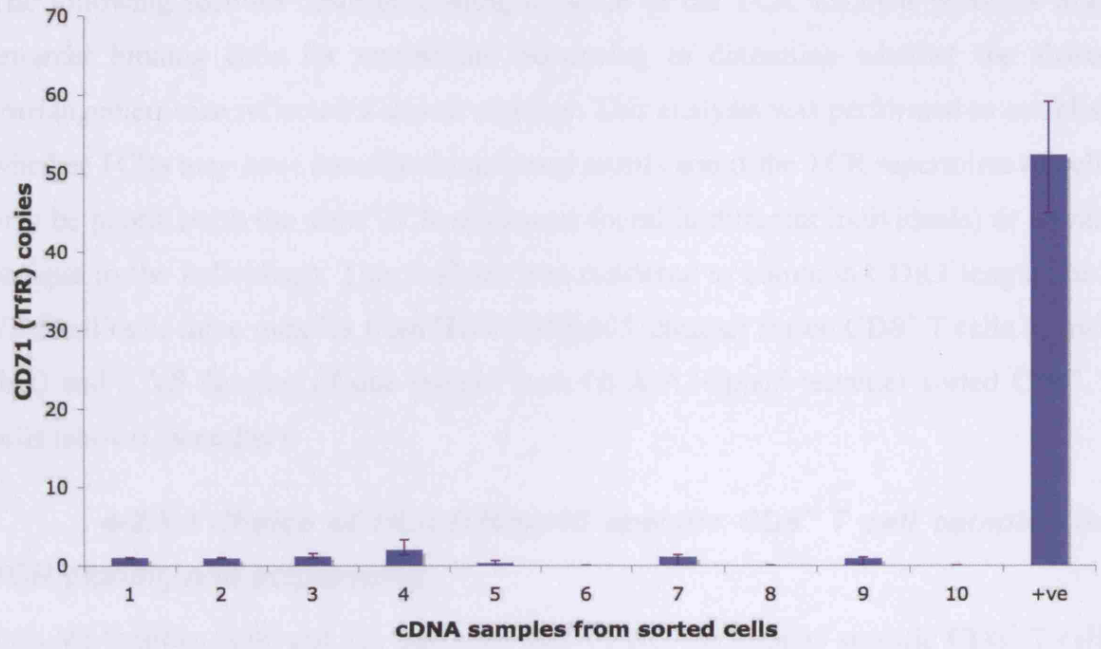


Figure 4-22 Quantification of a constitutive gene in cDNA from sorted CMV specific CD8$^+$ T cells that failed to produce detectable TCR Vß amplification signals

Transferrin receptor (TfR) transcripts were quantified in all cDNA samples that failed to produce detectable Vß PCR products (1-10) along with a positive control (+ve). The means of TfR copies detected in triplicate samples are shown. Errors represent the difference to the highest and lowest of the three values obtained respectively.

Figure 4-22 demonstrates that the constitutive gene was not or near to not detectable in cDNA samples for which spectratyping was not successful. This indicates that TCR analysis failed due to insufficient quantity or quality of cDNA obtained from cell samples. This maybe due to stress induced in cells when they were sorted off-site and stimulated before spectratyping.

4-2.5 TCR cloning and sequencing of CMV specific CD8$^+$ T cells

Spectratyping can be used for initial estimation of the TCR diversity of cells. However, it cannot define clonality, as it does not assess the extent of heterogeneity within a particular CDR3 TCR Vß length.

TCR repertoires of *ex vivo* CMV specific CD8$^+$ T cell responses with different HLA/CMV peptide target specificities were shown to be restricted to a few CDR3 size classes in HSCT patients in the previous sections. The detection of TCR Vß PCR products with similar CDR3 lengths, however, does not imply sequence similarity.

The following sections describe cloning of some of the TCR Vß PCR products from tetramer binding cells for nucleotide sequencing to determine whether the shared rearrangement size reflected a shared receptor. This analysis was performed to establish whether TCRs may have conserved junctional motifs and if the TCR repertoires of cells may be public (with the same TCR sequences found in different individuals) or private (unique to the individual). This analysis was restricted to common CDR3 lengths in 2 Vß families of three samples from HLA-B35/pp65 tetramer sorted $CD8^+$ T cells (shown first) and 2 Vß families of one sample from HLA-A24/pp65 tetramer sorted $CD8^+$ T cells (shown thereafter).

4-2.5.1 Choice of HLA-B35/pp65 specific $CD8^+$ T cell samples for TCR cloning and sequencing

Two Vß families (Vß6 and 12) that were used by HLA-B35/pp65 specific $CD8^+$ T cells from patients 4, 8 and 9 were chosen to further investigate shared class sizes.

Results from Vß12 spectratyping analysis of HLA-B35/pp65 specific $CD8^+$ T cells from patients 4, 8 and 9 are illustrated in Figure 4-23.

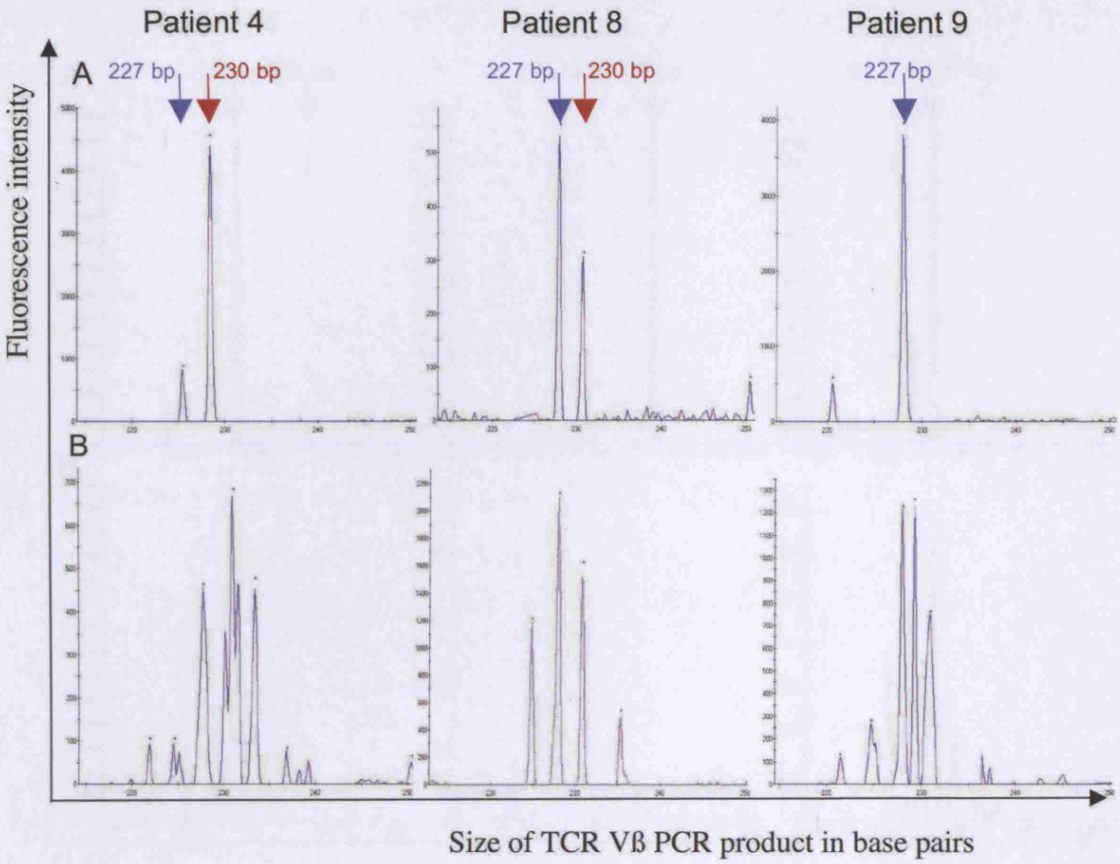


Figure 4-23 Shared TCR Vß12 size classes in patients 4, 8 and 9

Frequency histograms of TCR Vß 12 PCR products derived from cDNA from sorted cells from three patients. A: HLA/CMV peptide tetramer binding cell fraction; B: Cell fraction that did not bind HLA/CMV peptide tetramer.

Figure 4-23 illustrates that one Vß12 size class of 227 bp (indicated by blue arrow) was shared by patients 4, 8 and 9, whereas a second Vß12 size class of 230 bp (indicated by red arrow) was shared by patients 4 and 8. Vß PCR products of 227 bp demonstrated stronger fluorescent signals in cDNA obtained from the tetramer binding cell fraction than from the cell fraction that did not bind tetramer in patients 4 and 9. This was also the case for signals of the 230 bp PCR product from patient 4.

Results from Vß6 spectratyping analysis of HLA-B35/pp65 specific $CD8^+$ T cells from patients 4, 8 and 9 are illustrated in Figure 4-24.

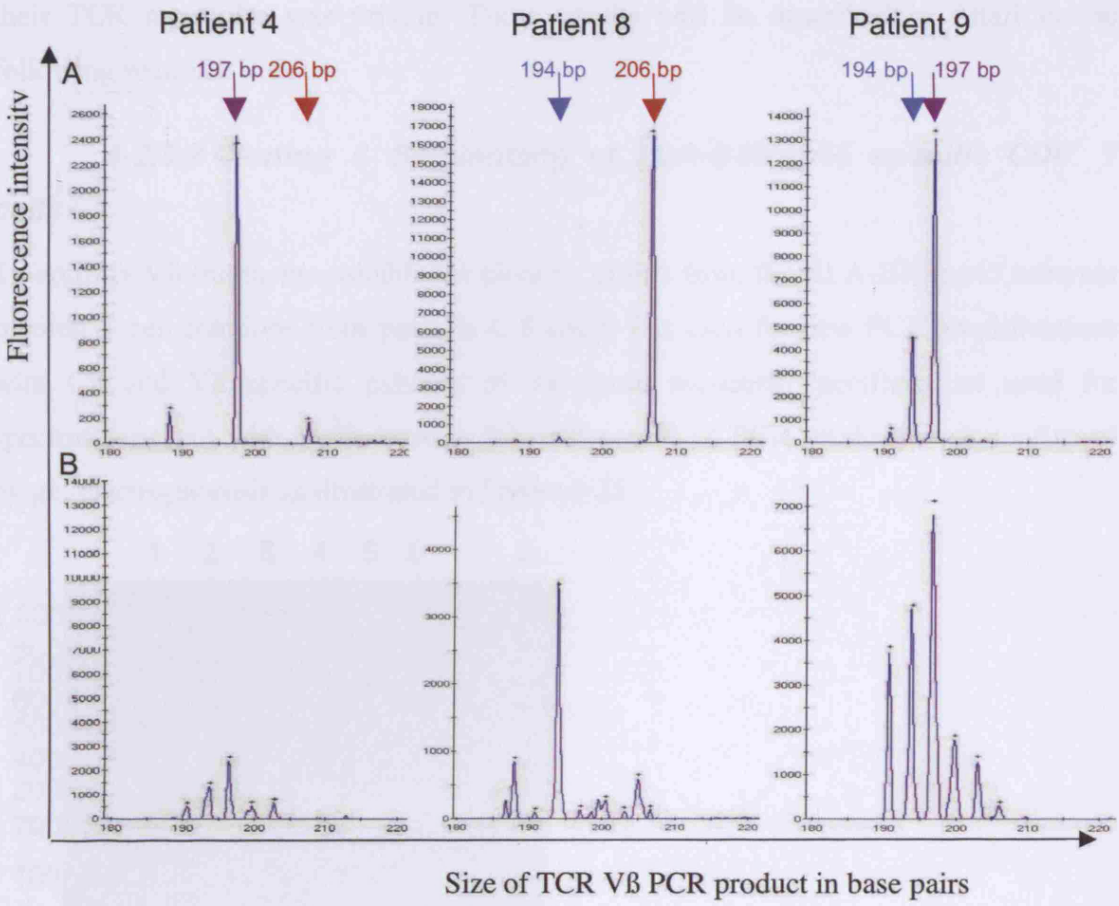


Figure 4-24 Shared TCR Vß6 size classes in patients 4, 8 and 9

Frequency histograms of TCR Vß 6 PCR products derived from cDNA from sorted cells from three patients. A: HLA/CMV peptide tetramer binding cell fraction; B: Cell fraction that did not bind HLA/CMV peptide tetramer. Shared CDR3 lengths are marked by arrows (purple: 197 bp, dark red: 206 bp).

Figure 4-24 illustrates that one Vß6 size class of 197 bp (indicated by purple arrow) was shared by patients 4 and 9. A second Vß6 size class of 206 bp (indicated by red arrow) was shared by patients 4 and 8. Both size classes demonstrated higher fluorescent signals in tetramer binding cell fractions than in cell fractions that did not bind tetramer. Patient 8 and 9 shared a third Vß6 size class of 194 bp (indicated by blue arrow). However, this size class did not demonstrate a higher fluorescent signal in tetramer binding cell fractions than in cell fractions that did not bind tetramer.

Vß 6 and 12 PCR products obtained from cDNA from tetramer binding cell fractions from patients 4, 8 and 9 were subsequently cloned and sequenced. This analysis demonstrated that patients shared only very limited junctional motifs within the size classes corresponding to spectratypes marked in blue in Figure 4-23 and Figure 4-24. However, T cell clones were rearranged from different TCR gene segments and

their TCR repertoire was private. These results will be described in detail in the following sections.

4-2.5.2 Cloning & Sequencing of HLA-B35/pp65 specific CD8⁺ T cells

To amplify Vß fragments suitable for cloning, cDNA from the HLA-B35/pp65 tetramer binding T cell fractions from patients 4, 8 and 9 was used for new PCR amplifications with Cß and Vß specific primers of the same sequence specificity as used for spectratyping but without fluorescent label. Presence of PCR products was confirmed by gel electrophoresis as illustrated in Figure 4-25.

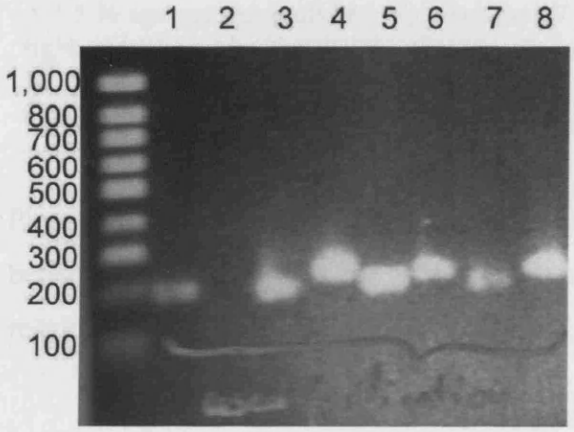


Figure 4-25 PCR amplification of TCR Vß fragments for bacterial cloning

A 1.5 % agarose gel is shown. Hyperladder IV (Bioline) was loaded in the far left lane with the molecular weight of the marker's standards (in bp) shown beside the gel picture. TCR PCR products from HLA-B35/pp65 tetramer binding cells were loaded in the remaining lanes (3: patient 4, Vß6; 4: patient 4, Vß12; 5: patient 8, Vß6; 6: patient 8, Vß12; 7: patient 9, Vß6; 8: patient 9, Vß12). PCR products from HLA-A24/pp65 tetramer binding cells from patient 11 (1: Vß7; 2: Vß20) are also shown. Cloning of these products is described in sections 4-2.5.4 and 4-2.5.5.

Bands of the expected size (182 - 248 bp, compare Table 2-7) were purified and ligated into TOPO TA pCR2.1 vector. *E.coli* cells were then transformed with the plasmids produced as described in section 2-13. Ten transformed colonies were picked for Vß6 cloning from patients 4 and 8; also ten colonies were picked for Vß12 cloning from patients 4 and 9. Fourteen transformed colonies were picked for Vß6 cloning from patient 9, and 14 colonies for Vß12 from patient 8. Plasmid DNA was extracted and the presence of TCR Vß inserts was confirmed by digestion of the vector with EcoRI followed by gel electrophoresis, which is illustrated in Figure 4-26.

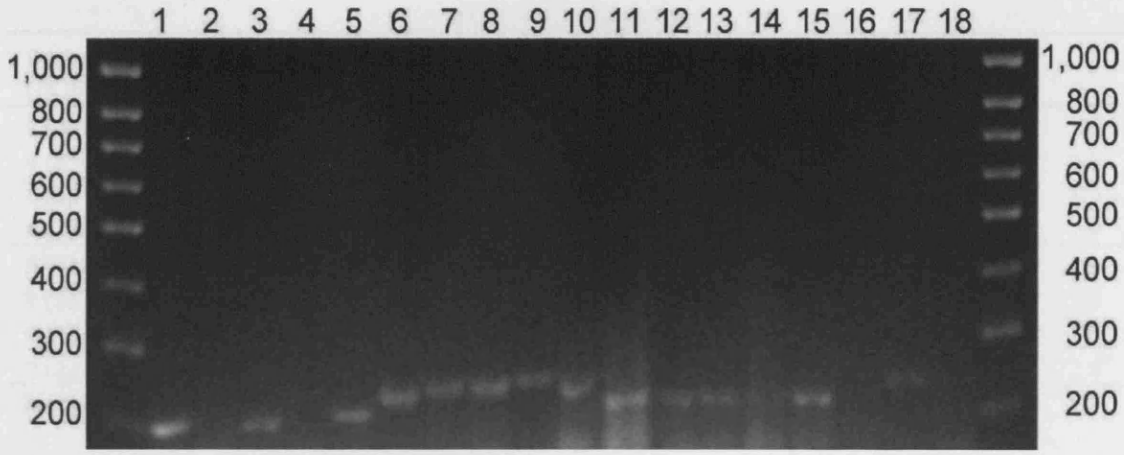


Figure 4-26 Confirmation of TCR inserts in plasmids from bacterial clones

A 1.5 % agarose gel is shown. Hyperladder IV (Bioline) was loaded in lanes on the far left and right end (the molecular weight of standards is shown in bp beside the gel picture). Lanes 1 to 18 contain representative samples of EcoRI digested plasmid DNA from bacterial TCR Vß6 (1-5, 11-15) and Vß12 (6-10, 16-18) clones.

Plasmids containing inserts of an expected size between 182 and 211 bp for Vß6 and between 219 and 248 bp for Vß12 were used for DNA sequencing. Good sequencing results were obtained and analysed from 6 to 13 clones from each TCR Vß cloning.

4-2.5.3 Junctional analysis of HLA-B35/pp65 specific CD8$^+$ T cells

Sequences obtained were aligned with the known sequence from the vector and those of the inserts were isolated. The codons obtained were translated into amino acid sequences.

IMGT/V-QUEST is a software program for T cell receptor V-J and V-D-J rearrangement analysis (Giudicelli *et al.*, 2004). It was used to analyse which V, D and J segments were used by TCR ß chains from tetramer binding CMV specific CD8$^+$ T cells and to determine whether TCRs of these clones may have conserved junctional motifs. As Vß primers used in this study are in accordance with the nomenclature by Wei and colleagues (Wei *et al.*, 1994) whereas IMGT/V-QUEST follows the IMGT TCR gene nomenclature (Lefranc, 2001a) the clones amplified with Vß6 and Vß12 primers translate into TRBV10 and TRBV7 sequences respectively.

A junctional analysis of TCR Vß12 clones identified in HLA-B35/pp65 tetramer binding cell fractions from patients 4, 8 and 9 is shown in Table 4-2.

TCR Vß12/TRBV10						
Patient (PCR size, bp)	**TRBV[1]**	**V(D)J**	**TRBD**	**TRBJ**	**CDR3 length**	**F**
4 (230)	10-1*01/02	CASNPGTGTDTQYF	1*01	2-3*01	12	5/9
4 (230)	10-1*01/02	CASNPGTGADEQYF	1*01	2-7*01	12	2/9
4 (227)	10-1*01/02	C**A**SNAGTGN**EQ**YF	1*01	2-7*01	11	1/9
4 (230)	10-1*01/02	CASNAGTGTDTQYF	1*01	2-3*01	12	1/9
9 (227)	**10-3***01/02/03/04	C**AIS**VAARG**EQ**F**F**	2*01	2-1*01	11	4/9
9 (233)	10-3*01/02/03/04	CAISETTRGDSPLHF	1*01	1-6*02	13	2/9
9 (233)	10-3*01/02/03/04	CAISXTTRGDSPLHF	1*01	1-6*02	13	1/9
9 (221)	10-1*01 02	[illegible]	-	1-2*01	NP	2/9
8 (227)	**10-3***01/02/03/04	C**AIS**GRGDT**EA**F**F**	1*01	1-1*01	11	8/8

Table 4-2 Junctional analysis of TCR Vß12 clones found in HLA-B35/pp65 specific CD8$^+$ T cells from patients 4, 8 and 9

This table lists the CDR3 sequences (V(D)J) and gene segments used by Vß12 clones. Similarities between sequences from different individuals are highlighted in bold. The frequency of clones detected is shown in column F. Differences in the nomenclatures used for Vß primers and in the analysis software result meant that clones amplified with Vß12 primers were assigned the name TRBV10. Two clones were found to have unproductively rearranged sequences due to out of frame junctional sequences (NP) and are shown in grey. [1] V gene segments from which sequences may have been rearranged could not be determined unambiguously due to short PCR products.

Table 4-2 demonstrates a restricted Vß12 diversity of TCRs of CMV specific CD8$^+$ T cells within each individual. This is especially pronounced in clones from patient 8, which all (n = 8) demonstrated the same sequence. PCR products amplified were too short to distinguish from which TRBV allele they may have been rearranged. Instead the IMGT blast search tool (http://imgt.cines.fr/blast/blast.html) determined several possible gene segments from which these sequences may have originated.

TCR rearrangements with a PCR product size of 230 bp (for which a major peak was observed in patient 4's spectratype and a small peak in patient 8's spectratype, compare Figure 4-23, page 300) were only detected in sequenced T cell clones from patient 4 but not patient 8.

TCR rearrangements with a PCR product size of 227 bp (detected in patient 4, 8 and 9's spectratypes, Figure 4-23) were detected in sequenced T cell clones from all three patients and sequence similarities of these are highlighted in bold in Table 4-2. Patients 8 and 9 shared only a minor junctional CDR3 motif (AIS) originating from TRBV10. Public TCRs were not observed and the receptors were rearranged from different TCR gene segments. A sequence alignment is illustrated in Figure 4-27. This figure shows a comparison of the Vß12 CDR3 sequences with a shared size class of

227 bp from patients 4, 8 and 9 by the ClustalW online tool, Version 1.83 (http://www.ebi.ac.uk/Tools/clustalw/index.html) (Chenna *et al.*, 2003). This is a multiple sequence alignment program that produces biologically meaningful illustrations of alignments of divergent sequences. Sequences are coloured crudely according to residue type as shown in Table 4-3.

```
A   Patient 4   CASNAGTGNEQYF
    Patient 8   CAISGRGDTEAFF
    Patient 9   CAISVAARGEQFF
                **  .     * :*

B   Patient 8   CAISGRGDTEAFF
    Patient 9   CAISVAARGEQFF
                ****  .  * **
```

Figure 4-27 ClustalW sequence alignment of CDR3 regions of TCR Vß12 clones with a PCR size of 227 bp found in HLA-B35/pp65 specific $CD8^+$ T cells from patients 4, 8 and 9

Alignment of amino acid sequences. Patients from whom sequences originate are indicated on the left. Symbols below the aligned sequences denote the degree of conservation observed at each position. The symbol "*" indicates that the residues or nucleotides in that column are identical in all sequences in the alignment. The symbol ":" indicates that substitutions have been observed, that are conserved according to Table 4-3. The symbol "." indicates that semi-conserved substitutions are observed.

Amino acids	Colour	Residue property
AVFPMILW	RED	Small and hydrophobic (inclusive aromatic -Y)
DE	BLUE	Acidic
RK	MAGENTA	Basic
STYHCNGQ	GREEN	Hydroxyl + Amine + Basic - Q
Others	Gray	-

Table 4-3 Colour code of protein sequence alignment

List of colours used to distinguish different amino acid residue types during ClustalW alignment.

Part A in Figure 4-27 demonstrates very limited similarity between aligned CDR3 sequences of TCR rearrangements with a PCR product size of 227 bp from patients 4, 8 and 9. Part B demonstrates that of the 11 amino acids located between the conserved cysteine and phenylalanine of the CDR3 region, 5 identical residues towards the ends of the sequence but only one semi-conserved substituted residue in the middle of the sequence can be observed between the aligned sequences from patients 8 and 9.

In addition to TCR rearrangements with PCR product sizes of 227 and 230 bp described above, there was a productively rearranged TCR with a PCR product size of 233 bp detected in sequenced T-cell clones from patient 9, which had not been detected by spectratyping. A TCR rearrangement with a PCR product size of 221 bp, which was detected in the spectratype of patient 9, was found to be a non-productive rearrangement when junctions of sequenced T-cell clones were analysed.

Junctional analysis of TCR Vß6 clones is illustrated in Table 4-4.

TCR Vß6/TRBV7

Patient (PCR size, bp)	TRBV[1]	V(D)J	TRBD	TRBJ	CDR3 length	F
4 (197)	7-3/4/6/7/8/9	CASSVRLAGA#YEQYF	2*01	2-7*01	NP (F)	5/6
4 (200)	7-3/4/6/7/8/9	VSLCQQLRSGRSG##EQYF	2*02	2-7*01	NP (F, S)	1/6
8 (206)	**7-4/6/7/8**	CASSLGAGTFKTNEKLFF	**1*01**	**1-4*01**	**16**	**9/11**
8 (194)	**7-3/6/7**	C**ASSL**TDGDQPQHF	**1*01**	**1-5*01**	**12**	**1/11**
8 (206)	7-3/4/6/7/8	VSLC*QLRGRDF*#TNEKLFF	1*01	1-4*01	NP (F, S)	1/11
9 (194)	**7-4/6/7/8/9**	C**ASSL**VASQETQYF	**2*01**	**2-5*01**	**12**	**1/10**
9 (197)	7-3/4/6/7/8	VSLCQHLPRGRV#ETQYF	2*01	2-5*01	NP (F, S)	5/10
9 (197)	7-3/4/6/7/8	CASIYRGAG*ETQYF	2*01	2-5*01	NP (S)	2/10
9 (194)	7-3/4/6/7/8/9	VSLCQQLSG*#QETQYF	2*01	2-5*01	NP (F, S)	1/10
9 (197)	7-3/4/6/7/8	VSLCQHLPRGRVRDPV#F	2*01	2-5*01	NP (F, S)	1/10

Table 4-4 Junctional analysis of TCR Vß6 clones found in HLA-B35/pp65 specific CD8$^+$ T cells from patients 4, 8 and 9

This table lists CDR3 sequences (V(D)J) and gene segments used by Vß6 clones. Similarities between sequences from different individuals are highlighted in bold. The frequency of clones detected is shown in column F. Differences in the nomenclatures used for Vß primers and in the analysis software result meant that clones amplified with Vß6 primers were assigned the name TRBV7. Clones with unproductively rearranged sequences (indicated as NP in the "CDR3 length" column) are shown in grey. "F" indicates that clones demonstrated out of frame junctional sequences, whereas "S" indicates stop codons. [1] V gene segments from which sequences may have been rearranged could not be determined unambiguously due to short PCR products.

A restricted diversity of TCRs of CMV specific CD8$^+$ T cells within each individual can also be observed for Vß6. Table 4-4 demonstrates a dominant sequence in 5 of 6 T-cell clones from patient 4, in 9 of 11 T-cell clones from patient 8 and in 5 of 10 T-cell clones from patient 9.

TCR rearrangements with a PCR product size of 206 bp (for which a major peak was observed in patient 8's spectratype and a very small peak in patient 4's spectratype, compare Figure 4-24, page 301) were only detected in sequenced T cell clones from patient 8 but not patient 4.

TCR rearrangements with a PCR product size of 197 bp found in spectratypes from patients 4 and 9 (Figure 4-24, page 301) were found to be non-productive rearrangements when junctions of sequenced T cell clones were analysed.

TCR rearrangements with a PCR product size of 194 bp detected in patient 8 and 9 by spectratyping (Figure 4-24) and T cell cloning shared a minor junctional CDR3 motif (ASSL) originating from TRBV7, which is highlighted in bold in Table 4-4. Public TCRs were not observed and T cell clones were rearranged from different TRBD and TRBJ gene segments. An alignment of CDR3 sequences of these TCR rearrangements is illustrated in Figure 4-28.

```
Patient 8  CASSLTDGDQPQHF
Patient 9  CASSLVASQETQYF
           *****. .:::.*:*
```

Figure 4-28 ClustalW sequence alignment of the CDR3 regions of TCR Vß6 clones with a PCR size of 194 bp found in HLA-B35/pp65 specific $CD8^+$ T cells from patients 8 and 9

This figure shows an alignment of amino acid sequences. Patients from whom sequences originate are indicated on the left. The meaning of symbols is as explained in Figure 4-27.

Figure 4-28 demonstrates only a limited degree of sequence similarity between aligned CDR3 sequences of TCR rearrangements with a PCR product size of 194 bp from patients 8 and 9. Of the 12 amino acids located between the conserved cysteine and phenylalanine of the CDR3 region, 5 identical residues can be observed at both ends of the sequence, whereas 3 conserved and 3 semi-conserved substituted residues can be observed in the middle of the sequences.

Additional small PCR amplifications of other sizes detected by spectratyping (for example a 188 bp amplification observed in patient 4 and 8, Figure 4-24) were not found by sequencing analysis of 6 to 11 T cell clones generated from each patient. Conversely, a non-productive TCR rearrangement with a PCR product size of 200 bp was observed by T cell cloning and sequencing from patient 4 although no amplification of the corresponding size was observed by spectratyping analysis.

These findings suggest that results from both methods, spectratyping and sequencing of bacterial TCR clones, can complement one another. Spectratyping results may need to be interpreted with care. They can indicate TCR rearrangements of similar CDR3 length in cells of different individuals. However, these could be non-productive rearrangements, as shown for Vß6 clones with a shared PCR size of 197 bp detected in

patients 4 and 9. Therefore spectratyping can provide a fast method to screen interindividual antigen specific cells for similarities of their TCR diversity and to determine which Vß families should be analysed in more detail. However, only junctional analysis of sequences from T cell clones can determine if TCR CDR3 lengths observed by spectratyping are from productively rearranged T cell receptor sequences.

All T cells have the potential to rearrange the TCRß locus on both chromosomes. TCR mRNA from a non-productively rearranged chromosome in some T cells can confuse the interpretation of spectratyping results. Here, non-productive Vß6 rearrangements with a PCR product size of 197 bp in patients 4 and 9 did not contribute to the TCR repertoire expressed on the surface of CMV specific $CD8^+$ T cells. They were present as mRNA, hitchhiking with the productively rearranged TCR mRNA that produced the antigen specific T cell receptor expressed on the surface of these cells.

4-2.5.4 Choice of HLA-A24/pp65 specific $CD8^+$ T cell samples for TCR cloning and sequencing

Along with HLA-B35/pp65 specific $CD8^+$ T cells from patients 4, 8 and 9 (compare section 4-2.5.1) HLA-A24/pp65 specific $CD8^+$ T cells from patient 11 were also used for further clonotypic analysis. Patient 11 demonstrated the highest frequency of HLA-A24/pp65 specific $CD8^+$ T cells (Figure 3-7, page 198). TCR PCR amplifications were observed in Vß 3, 4, 7, 14 and 20 from these cells.

Two PCR amplification peaks in Vß14 were either of lower signal strength in cDNA obtained from the tetramer binding cell fractions compared with the cell fractions that did not bind tetramer or below the background level of 100 fluorescence units. PCR amplifications from Vß3 and Vß4 demonstrated fluorescent peaks of only 100 fluorescence units, which is at the background limit of the assay. Therefore these signals were not considered to be genuine.

Fluorescent PCR signals in Vß7 demonstrated a single peak above background level, which was of equal strength in cDNA obtained from the tetramer binding cell fraction and from the cell fraction that did not bind tetramer. Signals from Vß20 demonstrated a peak above the background level in cDNA obtained from the tetramer binding cell fraction but not the cell fraction that did not bind tetramer. Results from Vß7 and 20 spectratyping analysis of HLA-A24/pp65 specific $CD8^+$ T cells from patient 11 are illustrated in Figure 4-29 and were chosen for further analysis.

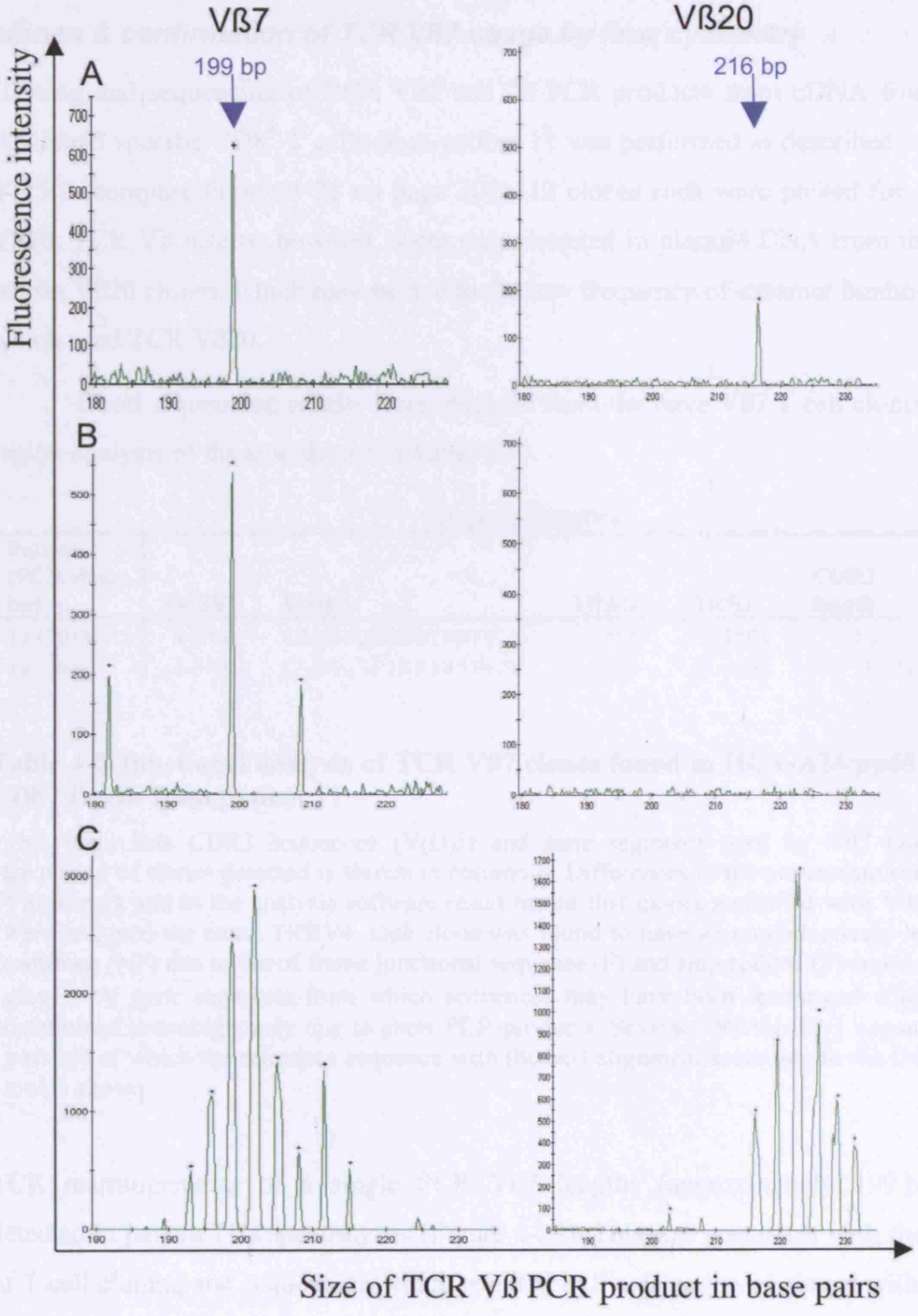


Figure 4-29 CDR3 size classes detected in TCR Vß7 and 20 from patient 11

Frequency histograms of TCR Vß7 (left) and Vß20 (right) PCR products derived from cDNA from sorted cells from patient 11 and a healthy control. A: HLA/CMV peptide tetramer binding cell fraction from patient 11; B: cell fraction that did not bind HLA/CMV peptide tetramer from patient 11; C: unsorted PBMC from healthy volunteer 19.

4-2.5.5 Junctional analysis of HLA-A24/pp65 specific CD8$^+$ T cell clones & confirmation of TCR Vß7 usage by flow cytometry

Cloning and sequencing of PCR Vß7 and 20 PCR products from cDNA from HLA-A24/pp65 specific CD8$^+$ T cells from patient 11 was performed as described in section 4-2.5.2 (compare Figure 4-25 on page 302). 12 clones each were picked for Vß7 and Vß20. TCR Vß inserts, however, were only detected in plasmid DNA from three Vß7 but no Vß20 clones, which may be due to the low frequency of tetramer binding T cells which used TCR Vß20.

Good sequencing results were obtained from the three Vß7 T cell clones. CDR3 region analysis of these is shown in Table 4-5.

	TCR Vß7/TRBV4					
Patient (PCR size, bp)	**TRBV[1]**	**V(D)J**	**TRBD**	**TRBJ**	**CDR3 length**	**F**
11 (201)	4-3*01	CASSPQSGLNTEAFF	1*01	1-1*01	13	2/3
11 (201)	4-3*01	CASSQDPIR##NTEAFF	1*01	1-1*01	NP (F, S)	1/3

Table 4-5 Junctional analysis of TCR Vß7 clones found in HLA-A24/pp65 specific CD8$^+$ T cells from patient 11

This table lists CDR3 sequences (V(D)J) and gene segments used by Vß7 clones. The frequency of clones detected is shown in column F. Differences in the nomenclatures used for Vß primers and in the analysis software result meant that clones amplified with Vß7 primers were assigned the name TRBV4. One clone was found to have an unproductively rearranged sequence (NP) due to out of frame junctional sequence (F) and stop codons (S) and is shown in grey. [1] V gene segments from which sequences may have been rearranged could not be determined unambiguously due to short PCR products. Several TRBV4-3/2/1 segments were possible of which the reference sequence with the best alignment according to the IMGT blast tool is shown.

TCR rearrangements of a single PCR Vß7 lengths (approximately 199 bp) were detected in patient 11's spectratypes (Figure 4-29). This was consistent with the finding of T cell cloning and sequencing which resulted in the detection of clones with roughly the same PCR product length (201 bp) in two productively rearranged Vß7 clones from patient 11 (Table 4-5). Similar to the results obtained from HLA-B35/pp65 specific CD8$^+$ T cells (4-2.5.3), there was also an unproductively rearranged clone detected in HLA-A24/pp65 specific CD8$^+$ T cells from patient 11. This sequence therefore did not contribute to the TCR repertoire expressed on the surface of CMV specific CD8$^+$ T cells.

The TCR usage of Vß7 and possibly Vß20 by HLA-A24/pp65 specific $CD8^+$ T cells from patient 11 was additionally tested by surface staining of tetramer binding cells with Vß7 and 20 specific monoclonal antibodies. Vß specifications allocated to these antibodies are as described in the nomenclature by Wei and colleagues (Wei *et al.*, 1994) and therefore correspond to the Vß specifications allocated to primers.

Staining for Vß20 was undetectable, which explains why TCR Vß inserts were not detected in plasmid DNA from Vß20 clones. HLA-A24/pp65 specific $CD8^+$ T cells had been isolated for spectratyping and cloning at day 588 post HSCT from patient 11. The next sample was obtained at day 602 at which time surface staining confirmed nearly 7 % of the tetramer binding population to be TCR Vß7. This is demonstrated in Figure 4-30 on the left.

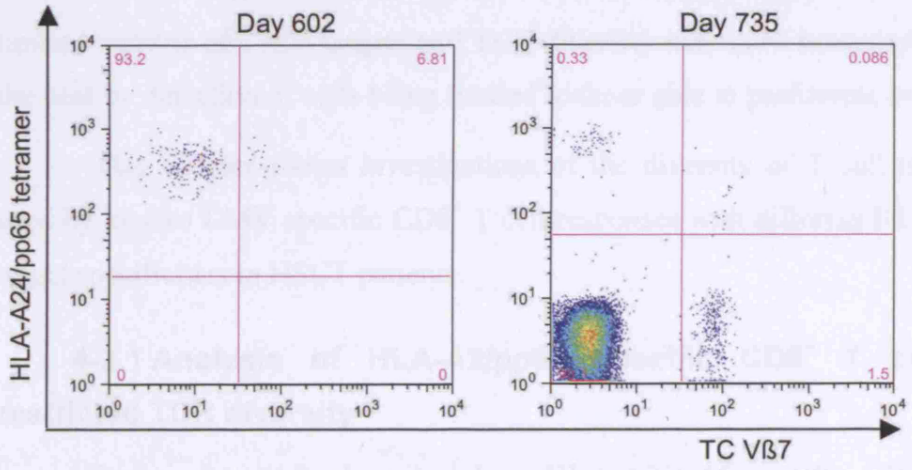


Figure 4-30 Staining of HLA-A24/pp65 specific $CD8^+$ T cells from patient 11 with anti-Vß7 monoclonal antibody

Tetramer binding cells from patient 11 at day 602 post transplant are shown on the left. The flow cytometry plot on the right shows $CD3^+$ $CD8^+$ live lymphocytes from patient 11 at day 735 post transplantation. Both plots are sorted according to their surface expression of TCR Vß7 on the x-axis and HLA-A24/pp65 tetramer staining on the y-axis.

This figure also demonstrates staining at a later time point, at which Vß7 staining was only observed in dim tetramer staining cells.

Patient 11 demonstrated a sharp increase in HLA-A24/pp65 tetramer binding $CD8^+$ T cells from day 532 post HSCT onwards (compare Figure 3-7 on page 198). Interestingly, Vß7 staining at several time points prior to day 532 was not detected in HLA-A24/pp65 tetramer binding $CD8^+$ T cells. This may well have been due to the lower frequency of HLA-A24/pp65 specific $CD8^+$ T cells, prohibiting detection of a fraction of these cells using TCR Vß7.

In conclusion the analysis of HLA-A24/pp65 specific $CD8^+$ T cells from patient 11 indicates that a considerable fraction of these cells used T cell receptors of the Vß7 family with the same CDR3 lengths. Vß7 usage was confirmed by surface staining of T cells, and T cell clones with identical CDR3 sequences were detected in that patient. However, TCR Vß7 was not identified in HLA-A24/pp65 specific $CD8^+$ T cells from two other patients from whom cells were used for spectratyping and therefore likely does not reflect a public T cell receptor.

4-3 Discussion

Cultured HLA-A2, HLA-B7 and HLA-B44 restricted CMV pp65 specific $CD8^+$ T cells were previously reported to use a restricted TCR repertoire (Peggs *et al.*, 2002, Weekes *et al.*, 1999b, Wills *et al.*, 1996). However, clonotypic analysis was restricted to a limited number of CMV targets and TCR diversity may have been underrepresented in the past by detection of cells being limited to those able to proliferate *in vitro*.

This chapter shows investigations of the diversity of T cell receptors (TCRs) used by *ex vivo* CMV specific $CD8^+$ T cell responses with different HLA/CMV peptide target specificities in HSCT patients.

4-3.1 Analysis of HLA-A2/pp65 specific $CD8^+$ T cells confirms restricted TCR diversity

During the study described here HLA-A2/pp65 specific $CD8^+$ T cells were analysed for comparison with CMV specific $CD8^+$ T cells targeting other HLA/peptide combinations. This analysis demonstrated that HLA-A2/pp65 specific $CD8^+$ T cells from 4 out of 5 patients used only 2 different Vß families whereas cells from the remaining patient used 10 (out of 24) Vß families. In comparison a previous study reported 2 to 9 clonotypes using comparative denaturing gradient gel electrophoresis (DGGE) analysis of HLA-A2/pp65 specific $CD8^+$ T cells that were FACS sorted from six nonagenarians and one middle aged healthy individual (Hadrup *et al.*, 2006). The report from that study did not list all Vß families in which clonotypes of CMV specific $CD8^+$ T cells were detected but demonstrated that most of them resided within Vß3.

The number of Vß families used by HLA-A2/pp65 specific $CD8^+$ T cells analysed in this project is lower than in previous reports that also used spectratyping and subsequent bacterial cloning of HLA-A2/pp65 specific $CD8^+$ T cells (Peggs *et al.*, 2002) but from eight healthy virus carriers. This is not surprising given the generally

lower TCR Vß diversity observed post transplantation in comparison to healthy individuals (Peggs *et al.*, 2003b, Verfuerth *et al.*, 2000). These studies reported that HLA-A2/pp65 specific $CD8^+$ T cells in healthy individuals used 5 to 20 Vß families (median 10). These included Vß1, 2, 4, 5, 6, 8, 9, 13, 14, 15 and 17 with major amplifications in Vß6, 8 and 13. Conservation of CDR3 lengths was not observed in that study, in which donor PBMC were cultured with autologous DC and CMV antigen *in vitro* (Peggs *et al.*, 2002).

In comparison, TCR Vß families that were used by HLA-A2/pp65 specific $CD8^+$ T cells of five HSCT patients in the project described here included Vß1, 4, 6, 8, 9, 12, 13S1, 14, 17, 20 and 24. The diversity of the TCR repertoire of these antigen specific cells was restricted to 1 or 2 different CDR3 lengths in most Vß families and did not exceed 4 different CDR3 lengths in any Vß family, similar to observations by Peggs and colleagues (Peggs *et al.*, 2002).

Another study using limiting dilution of NK cell depleted PBMC from six healthy virus carriers after stimulation with CMV infected fibroblasts demonstrated a more restricted TCR Vß usage than that observed here. That study reported one or two Vß families to be predominant in CMV specific $CD8^+$ T cells in each of the studied individuals. The authors used staining with Vß specific mAb and detected predominant Vß families within Vß6, 13.1, 14, 17, 20 and 22 (Wills *et al.*, 1996). The monoclonal antibody panel available at the time of that investigation recognised only a fraction of Vß families. However, a later study by the same group which analysed the TCR ß chain sequences of multiple independently derived CMV peptide specific $CD8^+$ T cell clones from six healthy virus carriers also demonstrated Vß gene usage to be restricted to one or two families in each individual which included Vß6, 7, 8, 9, 13.1, 14 and 17 (Weekes *et al.*, 1999b). The latter study also demonstrated a public Vß8 CDR3 sequence after stimulation with the HLA-A2 restricted pp65 peptide in two healthy donors.

These results were similar to the results of the study described here which also demonstrated shared CDR3 lengths in HLA-A2/pp65 specific $CD8^+$ T cells, which occurred in Vß6, Vß12 and Vß20 and may reflect public TCR repertoires.

Findings from previous reports on the TCR diversity of HLA-A2/pp65 specific $CD8^+$ T cells cited above analysed these cells after sampling from healthy virus carriers. A comparative analysis of HLA-A2/pp65 specific $CD8^+$ T cell clones expanded from five healthy virus carriers *versus* patients experiencing CMV reactivation (including seven rheumatoid arthritis patients and one and seven recipients of kidney or bone

marrow transplants respectively), however, demonstrated a TCR repertoire focusing upon chronic antigenic stimulation (Trautmann *et al.*, 2005). *Ex vivo* surface staining with monoclonal Vß specific antibodies during that study demonstrated the use of 2-5 Vß families in healthy virus carriers in comparison to the use of only one Vß family (Vß13S1) in the bone marrow transplant recipient. However, the authors acknowledged that the low frequency of HLA-A2/pp65 specific $CD8^+$ T cells likely influenced the accuracy of Vß surface staining of cells from that patient. That study found identical sequences in 4 Vß13S1 T cell clones derived from the bone marrow transplant patient and a highly restricted TCR repertoire in other patients experiencing CMV reactivation, in contrast to several different sequences in T cell clones derived from healthy virus carriers comprising the Vß families Vß 1, 2, 3, 6, 7, 8, 13S1, 14, 15, 20 and 23. Although the TCR CDR3 diversity detected in the study by Trautmann and colleagues may have been underrepresented by analysis of a limited number of T cell clones, it demonstrates that the clonotypic composition of HLA-A2/pp65 specific $CD8^+$ T cells from HSCT patients in the study described here can be expected to be more restricted than in reports on healthy virus carriers described above.

The reduction in TCR diversity in CMV reactivating patients compared to healthy virus carriers in the study by Trautmann and colleagues was associated with selection of clones with high affinity TCRs sharing public TCR Vα motifs. The same mechanism of focusing of a diverse primary CMV specific $CD8^+$ T cell response by selection of high affinity clones (Vß13.1 in HLA-A2/pp65 specific and Vß14 in HLA-B7/pp65 specific $CD8^+$ T cells) into the memory $CD8^+$ T cell pool was also observed by others (Day *et al.*, 2007). The focusing of the response observed by Day and colleagues was rapid, which may suggest that it was not driven by reactivation of the virus during long-term carriage (reviewed in (Waller *et al.*, 2008)). They used limiting dilution assays to generate biological T cell clones from three healthy virus carriers and two kidney transplant patients undergoing primary CMV infections. TCR Vß chains of the obtained T cell clones were sequenced to identify CDR3 diversity. PCR amplified cDNA prepared from tetramer sorted PBMC was used for bacterial cloning in parallel. Findings of the same sequences in multiple bacterial clones and biological T cell clones suggested that TCR CDR3 diversity may not be underrepresented by T cell cloning in comparison to *ex vivo* analysis of T cells.

In summary, the clonotypic analysis of HLA-A2/pp65 specific $CD8^+$ T cells in HSCT patients during this project resulted in findings comparable to previous reports.

Variations between any studies likely occur due to the dependence of detection of shared TCRs on the sampling effort and clonal dominance of these shared TCRs. Diversity was restricted to 1 or 2 CDR3 lengths in only 2 different Vß families in most patients, but inter-individual variability was evident by the observation of 11 different Vß families used within the patient cohort. The findings on TCR diversity of HLA-A2/pp65 specific $CD8^+$ T cells were used further for comparison with CMV specific $CD8^+$ T cells targeting other HLA/peptide combinations.

4-3.2 TCR diversity of $CD8^+$ T cells targeting various pp65 peptides is lower than that of $CD8^+$ T cells targeting pp50 (245-253)

HLA-A2, HLA-B7 and HLA-B44 restricted CMV pp65 specific T cells were reported to use a restricted TCR repertoire. However, the diversity of T cell receptors (TCRs) used by CMV specific $CD8^+$ T cell responses with other HLA/CMV peptide target specificities remained unclear when initiating this project and were therefore analysed in this study.

Initially, CMV specific $CD8^+$ T cells were sorted with immunomagnetic beads specific for the fluorochrome used for tetramer staining. The limited purity and low cell numbers obtained, however, necessitated a wide range of optimisation strategies for the separation of cells, as well as for subsequent steps to enable the analysis of the TCR diversity of *ex vivo* CMV specific $CD8^+$ T cells from patients. Results obtained with TCR activated cells that were FACS sorted and analysed using optimised cDNA generation, PCR and separation methods allowed for highly accurate clonotypic analysis with more reliable results than achieved initially.

The numbers of TCR Vß families found to be used by CMV specific $CD8^+$ T cells targeting various HLA/peptide combinations with these optimised methods are shown in Table 4-6.

$CD8^+$ T cell specificity	Average number of Vß families used
HLA-B35/pp65	2.0 (all 2, n = 3)
HLA-A24/pp65	1.5 (range 1-2, n = 2)
HLA-A1/pp65	5.0 (range 1-11, n = 3)
HLA-A1/pp50	9.5 (range 1-19, n = 4)
HLA-A2/pp65	3.6 (range 2-10, n = 5)

Table 4-6 Number of TCR Vß families used by TCRs of CMV specific $CD8^+$ T cells

The numbers of Vß families for which PCR amplifications were detected in cDNA from FACS sorted $CD8^+$ T cells binding to different tetramers are listed.

This table demonstrates that the TCR Vß diversity differed substantially between relatively similar (1.5 – 5 numbers of Vß families used on average) responses targeting different CMV pp65 peptides and responses targeting the peptide CMV pp50 (245-253) (9.5 numbers of Vß families used on average). To my knowledge, this is the first study to analyse the TCR repertoire diversity of $CD8^+$ T cells targeting a CMV pp50 epitope. The findings merit further investigation because they imply that it could be a general phenomenon that CMV pp50 specific $CD8^+$ T cells have a greater TCR repertoire than CMV pp65 specific $CD8^+$ T cells. Six additional CMV pp50 epitopes have been demonstrated to elicit $CD8^+$ T cell responses restricted by HLA-A1, HLA-A2, HLA-A3 or HLA-B44 (Elkington *et al.*, 2003). Analysis of the TCR diversity of $CD8^+$ T cells targeting these peptides may be informative to determine whether $CD8^+$ T cells targeting other CMV pp50 epitopes also use a less restricted TCR repertoire than those targeting CMV pp65, or whether the less restricted TCR repertoire is a property specific to CMV pp50 (245-253).

Studies on cellular responses to CMV pp50 have only recently emerged in the literature and knowledge of the immunogenicity of CMV pp50 is far more restricted than the knowledge of the properties of CMV pp65 (refer to section 1-11.2).

Since the TCR repertoire observed for $CD8^+$ T cells targeting CMV pp50 is less restricted, this suggests that focusing of a diverse primary CMV pp50 specific $CD8^+$ T cell response with selection of clones with high affinity TCRs into the memory $CD8^+$ T cell pool (as occurs with CMV pp65 (Day *et al.*, 2007)) may not occur. This remains to be investigated in future studies.

4-3.3 $CD8^+$ T cells for which higher cell numbers correlate with protection from CMV demonstrate greater TCR diversity

The number of TCR Vß families used by CMV specific $CD8^+$ T cells (Table 4-6) is more limited for cells targeting HLA-A24/pp65 (1.5 numbers of Vß families used on average) and HLA-B35/pp65 (2.0 numbers of Vß families used on average) than those targeting HLA-A2/pp65 (3.6 numbers of Vß families used on average) and HLA-A1/pp50 (9.5 numbers of Vß families used on average). In comparison, findings from the previous chapter demonstrated that CMV specific $CD8^+$ T cells targeting the first two mentioned HLA/peptide combinations inversely correlated with the ability to detect CMV reactivation at significantly lower levels (7.55 and 4.44 cells/µl of peripheral blood respectively) than those targeting the latter HLA/peptide combinations (13.23 and 17.15 cells/µl of peripheral blood respectively). These observations suggest that $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation at high cell levels appeared to use highly diverse T cell receptors, whereas $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation at low cell levels appeared to use less diverse T cell receptors. To address whether this finding may be a sampling artefact, cell numbers used for spectratyping were directly compared with spectratyping results (Figure 4-20) demonstrating no direct correlation.

Both cell levels that correlated with protection from CMV and the number of Vß families used by CMV specific $CD8^+$ T cells of certain HLA/peptide specificities, demonstrated substantial variability between individual patients. Most samples used for clonotypic analysis were derived from patients that were not included in the follow-up cohort (Table 2-1, page 99) which was used to determine what cell numbers inversely correlated with the ability to detect CMV reactivation in Chapter 3. Those that were ($n = 5$) are shown in Table 4-7, which lists the cells numbers that inversely correlated with the ability to detect CMV reactivation and the number of Vß families used by CMV specific $CD8^+$ T cells in these patients.

Patient	$CD8^+$ T cell specificity	Number of Vß families used	Protective cell level
1	HLA-B35/pp65	2	1.46
2	HLA-A24/pp65	2	6.77
21	HLA-A1/pp50	1	14.20
14		13	16.67
22		5	24.46

Table 4-7 Number of TCR Vß families used by CMV specific $CD8^+$ T cells in comparison to their cell levels correlating with protection from CMV in individual patients

The numbers of Vß families for which PCR amplifications were observed from cDNA from $CD8^+$ T cells binding to different tetramers (specificity listed in the second column) and the concentration of cells (cell number/µl blood) observed to correlate with protection of CMV in individual patients ("protective levels") are listed.

This table illustrates a general trend towards positive correlation between both factors and inter-individual variability.

4-3.4 The TCR sequences used by CMV specific $CD8^+$ T cells are private

Spectratyping analysis estimated a restricted use of TCRs by CMV specific $CD8^+$ T cells. To assess the extent of heterogeneity within particular TCR Vß size classes, cloning and sequencing of TCRs was performed for a subset of HLA-B35/pp65 and HLA-A24/pp65 specific $CD8^+$ T cells.

Junctional analysis of CMV specific $CD8^+$ T cells was until recently confined to HLA-A2, HLA-B7 and HLA-B44 restricted responses (Day *et al.*, 2007, Khan *et al.*, 2002a, Khan *et al.*, 2002b, Peggs *et al.*, 2002, Trautmann *et al.*, 2005, Weekes *et al.*, 1999b). Findings from this project demonstrated dominant clones with conserved CDR3 lengths in HLA-B35/pp65 specific $CD8^+$ T cells. These cells demonstrated only very limited junctional similarity and no use of public TCR for Vß6 (TRBV7) and Vß12 (TRBV10).

This is in contrast to results from the first study on HLA-B35/pp65 specific $CD8^+$ T cells that was published recently. In contrast to results from this project, that study reported skewing of the TCR repertoire of T cells towards TCR Vß3 (TRBV28) in four of five healthy virus carriers, with surface expression ranging from 19 % to 55 % (average 34 %) when measured by tetramer and Vß antibody staining (Brennan *et al.*, 2007). Further clonal analysis by Brennan and colleagues demonstrated a conserved

CDR3 length of 13 residues that was exclusively paired with TRBJ1-4, with a public TCR Vß sequence in 2 donors. Two public TRAV17/TRAJ33 sequences were shared in three donors each.

In comparison, TCR Vß3 (TRBV28) amplifications of a 200 bp PCR product were shared in the spectratypes of four of the six patients analysed within this project. However, as HLA-B35/pp65 specific $CD8^+$ T cells of three of these patients (patients 4, 8, and 9) were sorted with beads (resulting in lower purity of cells before the optimised FACS method was used), PCR amplifications of TCR Vß sequences in these cells were not considered genuine if the fluorescence intensity signals in tetramer binding cells was lower than the signal strength observed in cells that did not bind tetramer, which was the case for patients 4 and 9 (compare section 4-2.2.5). Accordingly the shared size class detected for Vß3 was not considered for further analysis.

Results from Vß3 spectratyping of HLA-B35/pp65 specific $CD8^+$ T cells in patients 4, 8, 9 and 35 are summarised in Table 4-8.

Patient	Vß3 size class (in bp)	Fluorescence intensity detected
4	197	1,500 *
	200	700 *
	209	250 *
8	200	4,000
9	188	1,400 *
	194	300 *
	197	300 *
	200	800 *
	209	250 *
35	200	500

Table 4-8 Vß3 size classes detected by spectratyping of HLA-B35/pp65 specific $CD8^+$ T cells in patients 4, 8, 9 and 35

List of TCR Vß3 size classes detected in cDNA from tetramer binding cells that were sorted with MACS beads from patients 4, 8 and 9 or by FACS in patient 35. "*" indicates size classes in MACS sorted tetramer binding cells for which the fluorescence intensity was less than that observed in cells that did not bind tetramer.

The fluorescent strength of all Vß3 PCR products with a length of 200 bp detected in these patients was above the detection limit (>100 fluorescent units) therefore confirming TCR Vß3 (TRBV28) usage of HLA-B35/pp65 specific $CD8^+$ T cells in the majority of the patient cohort within this study.

However, in contrast to the findings of Brennan and colleagues, observations from this study do not suggest skewing of the TCR repertoire of HLA-B35/pp65

specific $CD8^+$ T cells towards TCR Vß3 (TRBV28). The highest amplification signals were detected for TCR Vß12 in patient 4 (signal strength more than 6x higher than for Vß3 PCR products of 200 bp length), for Vß6 in patient 8 (signal strength more than 4x higher than for Vß3 PCR products of 200 bp length) and for Vß14 in patient 9 (signal strength 25x higher than for Vß3 PCR products of 200 bp length). In patient 35, the highest TCR amplification signal was detected for TCR Vß3 and signals were detected in only one other Vß family, which was Vß8 (compare Figure 4-18 in section 4-2.2.5 on page 291). Low frequency T cell receptor rearrangements may be efficiently amplified by PCR. Therefore spectratyping results obtained from tetramer sorted cells in this study cannot be used to draw conclusions on the frequency of Vß size classes within the analysed cell samples. Nevertheless, from the findings described above it is clear that HLA-B35/pp65 specific $CD8^+$ T cells from the patients analysed in this study used several TCR Vß families and Vß3 was not used in all of the patients (not in patients 1 and 31). Therefore skewing of the TCR repertoire of HLA-B35/pp65 specific $CD8^+$ T cells towards TCR Vß3 was not confirmed in this study.

To obtain a better understanding of how frequent particular Vß size classes may be within the $CD8^+$ T cell population, future studies could make use of internal standards. Pannetier and colleagues argued that Vß-Cß PCRs are competitive amplifications of sequences that are nearly identical except for the CDR3 part of their sequences, which should allow the PCR to be run in saturation while remaining quantitative if internal standards are used during amplification (Pannetier *et al.*, 1992, Pannetier *et al.*, 1993).

In addition to analysis of HLA-B35/pp65 specific $CD8^+$ T cells, junctional analysis was also performed on HLA-A24/pp65 specific $CD8^+$ T cells from patient 11 for TCR Vß7. Surface staining of HLA-A24/pp65 specific $CD8^+$ T cells with anti-Vß antibody in this patient confirmed the findings from TCR cloning, which suggested that only a single Vß family was used. Surface staining also demonstrated that TCR Vß usage changed over time suggesting that the clonality of *ex vivo* CMV specific $CD8^+$ T cells is diverse both between different individuals and at different times within the same patients.

4-3.5 Limitations of the clonotypic TCR Vß analysis

The clonotypic analysis of CMV specific $CD8^+$ T cells performed in this study was limited by detection of PCR amplification of the TCR in only 68 % of samples analysed. Findings were, however, verified by quantification of a constitutive gene, which indicated that failure of PCR product detection was due to low quantity/quality of the cDNA in the remaining samples (Figure 4-22, section 4-2.4, page 298) rather than inherent to the procedure. This may have been a result of cell stress induced by the transport of cells required by off-site FACS sorting or the subsequent stimulation of cells to induce TCR mRNA levels before PCR.

As discussed in section 4-3.3, observations from this study suggest that $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation at high cell levels appeared to use highly diverse T cell receptors, whereas $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation at low cell levels appeared to use less diverse T cell receptors. It could be argued that a lower frequency of cells may enable selection of a lower number of cells resulting in detection of less TCR Vß families in those cells due to the detection limits of the assays. This potential artefact was further analysed by plotting the numbers of Vß families detected by clonotypic analysis against the numbers of cells originally used for that analysis. Although this approach cannot rule out a biased detection of TCR Vß families in those CMV specific $CD8^+$ T cells available in low numbers, the plot of the two variables (Figure 4-20 in section 4-2.3, page 295) demonstrated no overall correlation of the two parameters. Results suggest that detection of different numbers of Vß gene families was dependent on HLA/peptide specificity of cells rather than the quantity of cells used for the analysis.

A third observation stood out to be a potential artefact. TCR usage of most CMV specific $CD8^+$ T cells was detected in Vß families 6 and/or 12, which may have resulted from variable efficiencies of primers specific for these Vß families. However, Rosenberg and colleagues previously used a method that avoids any potential bias introduced by varying efficiencies of different Vß primers. Their findings demonstrated the preferential usage of Vß4, 6 and 12 amongst 250 sequences from peripheral T cells from 5 healthy volunteers (Rosenberg *et al.*, 1992). A biased use of Vß6 and 12 families in the peripheral repertoire may therefore explain the higher probability of these Vß families being used by peripheral T cells of any specificity.

The clonotypic analysis performed in this study was also limited by the sorting purity that could be achieved by MACS bead sorting. This may have affected the accuracy of spectratyping results. This was addressed by the development of enhanced cell preparation - and spectratyping methods including the use of FACS sorting. Better interpretation of spectratyping results may also be achieved by the use of PCR standards (compare section 4-3.4) or by additional surface staining of cells with anti-Vß antibodies, to determine the frequency of each TCR Vß family within the cell population that is studied. This may amend spectratyping results in future studies since PCR (used in the latter) can amplify targets irrespectively of their frequency in the original population.

4-3.6 Implications of findings and final conclusions

The clonality of CMV specific $CD8^+$ T cells present at a significant frequency in peripheral blood has a wide-ranging impact on patients. As will be outlined below, CMV drives the expansion of $CD8^+$ T cell subsets that not only correlate with protection against CMV but that are also linked to immunosenescence suggesting that the virus accelerates the process of age-associated deterioration of immune functions.

Higher levels of CMV specific $CD8^+$ T cells were observed in HSCT patients than in healthy virus carriers in the previous chapter (refer to Figure 3-28, page 246), which was attributed to the homeostatic drive, in addition to CMV antigen stimulus in conditioned patients. By aiming to contain the virus (that cannot be completely eliminated once it has infected the human host), the immune response leads to the accumulation of highly expanded oligoclonal CMV specific $CD8^+$ T cells that result in detrimental effects, with changes similar to those seen in an aged immune system.

Measurement of the TCR diversity of CMV specific $CD8^+$ T cells in HSCT patients may have several important implications. On the one hand, the knowledge of public TCRs used in the T cell response to different CMV antigens may be of clinical use for monitoring of CMV responses in high-risk immunodeficient patients. In this regard the use of clonotypic probes may be useful for enhanced detection of CMV specific $CD8^+$ T cells. It may also be useful for the development of treatment modalities using TCR gene transfer. On the other hand, reports summarised below suggest that the highly restricted TCR usage of CMV specific $CD8^+$ T cells in HSCT patients may be a marker of detrimental effects on the $CD8^+$ T cell pool that accelerate the process of age-

associated deterioration of immune functions and render the patient susceptible to other infections.

Clonal T cell expansions are common in the elderly and can occupy a large proportion of the T cell pool (Posnett *et al.*, 1994). These expansions are commonly highly differentiated effector memory $CD8^+$ T cells of $CD28^-$ phenotype and result in a change of the CD4/CD8 ratio, which was termed the immune-risk phenotype (IRP) because of its association with increased mortality in 80 and 90 year olds (Wikby *et al.*, 2002).

The IRP was associated with CMV seropositivity. It was shown that CMV specific $CD8^+$ T cells accumulate during CMV latency over time (Karrer *et al.*, 2003, Komatsu *et al.*, 2003) and that these are highly differentiated $CD8^+$ T cells of a $CD28^-$ phenotype (Vescovini *et al.*, 2004). In elderly CMV carriers and HSCT patients, the substantial accumulation of CMV specific $CD8^+$ T cells is linked to the development of large clonal expansions (Khan *et al.*, 2002b). This memory inflation contributes to early immunosenescence. Previous reports (using tetramer staining on samples from a large number of subjects and multivariate regression analysis) demonstrated CMV seropositivity being associated with a marked effect on the overall phenotype of $CD8^+$ T cells (at any given age) and estimated an apparent "ageing effect" of CMV on $CD8^+$ T cells of 35.4 years (Northfield *et al.*, 2005). This can be explained as follows.

The high immunological investments against CMV may divert finite immunological resources away from other potential antigenic challenges and thereby negatively influence the immune fitness of patients by reducing the overall level of antigenic diversity. Shrinkage of the TCR repertoire may render individuals susceptible to infections other than CMV (Hadrup *et al.*, 2006). Furthermore the number of dysfunctional CMV specific $CD8^+$ T cells that do not respond to specific antigen *ex vivo* and are considered to be anergic was reported to be increased in the elderly (Ouyang *et al.*, 2003, Ouyang *et al.*, 2004). Dysfunctional CMV specific $CD8^+$ T cells were associated with a highly differentiated ($CD28^-$) and killer cell lectin-like receptor G-1 $(KLRG1)^+$ $CD57^+$ phenotype (Wikby *et al.*, 2002) that was initially linked to reduced proliferative capacity (Ibegbu *et al.*, 2005). More recently, however, the $CD28^-CD57^+$ phenotype was reported to represent a highly differentiated T cell subset with unique functional features, well capable of proliferation (Chong *et al.*, 2008). Hadrup and colleagues found that the variety of CMV specific T cell clones increases with age but

declines in the final years of IRP individuals correlating with survival time (Hadrup *et al.*, 2006).

The previous and this chapter showed that $CD8^+$ T cells directed towards multiple CMV antigens inversely correlated with the ability to detect CMV reactivation at different levels and varied phenotypically and in their T cell receptor diversity. These results suggest that broadly directed $CD8^+$ T cell responses may carry out divergent functions. Therefore, $CD8^+$ T cell responses targeting many different CMV epitopes may be essential in controlling CMV replication. Consistent with this hypothesis, it was suggested that infected individuals with broad T cell reactivity were able to clear virus infections more efficiently than those with a more narrowly focused T cell response (Thimme *et al.*, 2001). Findings from this project further the prospects of the development of prophylactic and therapeutic anti-CMV treatments by providing the knowledge needed to monitor the efficiency of treatments comprising HLA-A1/pp50, HLA-A2/pp65, HLA-A24/pp65 and HLA-B35/pp65 $CD8^+$ T cell responses. Data also provide further insight into the complexity of cellular immune responses to CMV in patients.

In summary, this chapter showed that the TCR diversity of *ex vivo* CMV specific $CD8^+$ T cells targeting different pp65 peptides presented by a variety of HLA is similarly restricted to those targeting HLA-A2/pp65 and HLA-B7/pp65 specific $CD8^+$ T cells studied previously. Findings demonstrated a restricted number of TCR Vß families with conservation of CDR3 lengths but no public TCR used by $CD8^+$ T cells targeting HLA-A1/pp50, HLA-A1/pp65, HLA-A24/pp65 and HLA-B35/pp65. Observations suggest that $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation at high cell levels appeared to use highly diverse T cell receptors, whereas $CD8^+$ T cells that inversely correlated with the ability to detect CMV reactivation at low cell levels appeared to use less diverse T cell receptors. However, heterogeneous TCR usage between cells from different individuals or between cells analysed at different time points in an individual is evident from this and other studies (Hadrup *et al.*, 2006). TCR Vß restriction differed substantially between $CD8^+$ T cells targeting CMV pp65 peptides and $CD8^+$ T cells targeting the peptide CMV pp50 (245-253). This study represents the first clonal investigation of HLA-A1/pp50 specific $CD8^+$ T cells. Due to their high TCR diversity, these cells may confer an advantage to patients in that they may result in a slower drive of immunosenescence than CMV specific $CD8^+$ T cells with more restricted TCR repertoires, which merits further study.

CHAPTER 5 GENERAL DISCUSSION

Current antiviral therapies are limited in that they only target replicating but not latent virus, are associated with significant toxicity and are counteracted by the occurrence of resistant viral strains. Alternative approaches, such as an adoptive therapy, are needed to combat the development of potentially fatal CMV related complications in immunosuppressed patients. The safety and proof of principle of such a therapy has been demonstrated in patients reconstituting their immune system after haematopoietic stem cell transplantation (HSCT) some years ago (Walter *et al.*, 1995).

The successful generation of HLA-A2/pp65 specific $CD8^+$ T cells under GMP conditions has already been demonstrated (Li Pira *et al.*, 2006) and recovery of between $1x10^7$ (Cwynarski *et al.*, 2001) and $2x10^7$ (Aubert *et al.*, 2001) of these cells/L of blood was reported to correlate with protection against CMV in patients.

Results presented in Chapter 3 demonstrate the successful detection and monitoring of different CMV specific $CD8^+$ T cell populations. Numbers of $CD8^+$ T cells against CMV peptides presented by the common HLA-A*0101, HLA-A*2402 and HLA-B*3501 alleles that inversely correlated with the ability to detect CMV reactivation in HSCT patients had not been shown previously. Results from this study demonstrate significant differences between levels of these different co-dominant CMV specific $CD8^+$ T cells that, in the presence of $CD4^+$ T cell help, inversely correlate with the ability to detect CMV reactivation. These differences are also apparent in measurements of those cells in healthy donors. Recovery of CMV specific $CD8^+$ T cells was shown to reach frequencies of up to 21 % (Cwynarski *et al.*, 2001) and 40 % (Ozdemir *et al.*, 2002) of all $CD8^+$ T cells in HSCT patients in previous studies. This study demonstrates an even higher percentage of CMV specific $CD8^+$ T cells reaching up to 69.4 % of all $CD8^+$ T cells in one patient (patient 29).

The long follow-up period of some patients within this study revealed delayed detection of CMV replication and CMV immune responses in patients receiving prolonged immunosuppression, which is especially pronounced post reduced intensity conditioning beyond the limit of one year post HSCT reported previously (Junghanss *et al.*, 2002). The detection of CMV replication after 500 days post transplantation and initial detection of CMV specific $CD8^+$ T cells at such a late time in one patient (patient 11) suggests that prolonged surveillance of viral replication and immune status in

patients receiving reduced-intensity conditioning may be advantageous beyond one year.

Cells were studied by the use of HLA/peptide tetramer complexes, which act as surrogates for HLA/peptide ligands on the surface of antigen presenting cells (to which T cells would respond naturally) and visualise T cells according to their antigen specificity. Staining with HLA/peptide tetramers is highly specific (Burrows *et al.*, 2000). Accordingly tetramer guided selection and subsequent adoptive transfer of cells may be especially useful in the HSCT setting, where non-specific T cells can cause GvHD. Despite the high specificity of tetramer complexes, the possibility of crossreactivity of cells remains since redundancy is an intrinsic property of TCR recognition (Mason, 1998). Whereas occurrence of cross-reactive CMV specific $CD8^+$ T cells was suggested in recipient$^-$/donor$^+$ HSCT pairs previously (Cwynarski *et al.*, 2001, Gandhi *et al.*, 2003a), this study presents, for the first time, a recipient$^+$/donor$^-$ HSCT case where development of cross-reactive CMV specific $CD8^+$ T cells is suspected (patient 7; refer to section 3-2.1.3, page 182).

However, this phenomenon is thought to be a rare event and availability of GMP grade tetramer sorted CMV specific $CD8^+$ T cells may help to provide therapy for many patients if adoptive therapy trials can confirm the protective capacity of these cells *in vivo*. The knowledge of protective levels of CMV peptide specific $CD8^+$ T cells restricted by different HLA alleles is important to enable monitoring of the effectiveness of treatment modalities in patients who are likely to express a variety of HLA tissue types. The quantity of cells that were shown to inversely correlate with the ability to detect CMV reactivation in patients in this study could be used as a marker for monitoring patients' immune status towards CMV even if these cells are not protective *per se*.

Results presented in Chapter 3 clearly show that CMV specific $CD8^+$ T cell numbers only inversely correlated with the ability to detect CMV reactivation in patients that recovered at least 160 $CD4^+$ T cells/µl blood. $CD8^+$ T cells in any given individual can target at least 6 major HLA alleles, which can potentially bind many different CMV derived peptides. Therefore measurements using a limited panel of tetramers are only informative of protective levels if they reveal responses to the target specificity analysed. This explains recently published results demonstrating that monitoring CMV specific $CD8^+$ T cell using a single tetramer (HLA-A2/pp65) cannot predict CMV reactivation (Morita-Hoshi *et al.*, 2008). In my opinion these findings

should not be interpreted as a lack of protective capacity of CMV specific $CD8^+$ T cell. Results from this and other studies show that, provided that $CD8^+$ T cells targeting a specific CMV epitope can be measured in the presence of $CD4^+$ T cells in patients, their numbers inversely correlate with the ability to detect CMV reactivation. Therefore the application of a wide range of tetramer reagents should allow for determination of a subgroup of patients who could be spared antiviral treatment (and its associated toxicity). Also adoptive therapy may help to prevent the development of CMV disease in the remaining patients with relevant HLA types.

The high specificity of HLA/peptide tetramers constitutes great advantages to their use but also limits their application to individuals possessing the relevant HLA molecules. This may be overcome to some extent by the selection of peptides presented by a variety of HLA alleles that are common in major ethnic populations. Previous studies suggested that 90 % coverage of some ethnic groups might be attainable with as few as eleven $CD8^+$ T cell epitopes (Longmate *et al.*, 2001). Adoptive transfer of $CD8^+$ T cells against CMV peptides presented by the common HLA-A*0101, HLA-A*0201, HLA-A*2402 and also HLA-B*3501 may enable therapy of more than three quarters of European Caucasoids and many Asians (compare section 1-11.1).

Many new CMV epitopes have been discovered during the last years (Elkington *et al.*, 2003, Burrows *et al.*, 2007, Khan *et al.*, 2007). This information could be used to widen the application of adoptive therapy to more patients of various other ethnic groups. Findings of an increasing number of CMV epitopes that are targeted during the naturally occurring T cell response to CMV raise the question of how diverse the total T cell response to CMV might be and how immunogenic each of the epitope specific T cell responses within that total T cell response might be. If adoptive therapy is to efficiently protect immunocompromised patients, these questions need to be addressed.

CMV specific $CD8^+$ T cells with high T cell receptor diversity may counter immune escape mechanisms and contribute only minimally to CMV associated immunosenescence, thus providing an advantage over CMV specific $CD8^+$ T cells with less T cell receptor diversity. The clonality of T cells may therefore aid to choose the T cells that are most likely to efficiently protect and benefit patients from amongst T cells targeting all of the known CMV epitopes.

Findings presented in Chapter 4 demonstrate significant differences between the diversities of TCRs used by the different CMV specific $CD8^+$ T cells isolated from HSCT patients. This study is the first to describe TCR diversity of $CD8^+$ T cells

targeting a CMV pp50 epitope. Interindividual heterogeneity was evident but overall it was found that $CD8^+$ T cells targeting HLA-A1/pp50 used significantly more diverse TCRs than $CD8^+$ T cells targeting different CMV pp65 epitopes presented by HLA-A1, HLA-A2, HLA-A24 or HLA-B35.

The CMV pp50 protein has not been studied extensively as a target for cytotoxic T cells previously. Since findings presented here demonstrate an exceptionally high TCR diversity of T cells targeting one of the epitopes of the CMV pp50 protein, therapeutic use of those cells may decrease the likelihood of CMV immune escape in patients. Due to their high diversity of T cell receptors, $CD8^+$ T cells targeting this epitope may also have less impact on the drive of immunosenescence than CMV specific $CD8^+$ T cells with more restricted TCR repertoires. The CMV pp50 protein therefore represents an interesting antigen to be investigated in future studies to establish whether other epitopes derived from this protein may also elicit T cell responses with T cell receptors that are more diverse than those found in CMV pp65 specific $CD8^+$ T cells.

CMV specific $CD8^+$ T cells were long thought to target mainly the CMV pp65 protein, which was thought to be related to the downregulation of class I HLA as one of the immune escape strategies of CMV that prevents the presentation of proteins, which are not introduced during viral penetration (before the onset of expression of viral immune escape genes) like pp65. More recent reports, however, demonstrate that $CD8^+$ T cell responses to CMV often contain multiple antigen reactivities, which are not constrained to pp65 (Elkington *et al.*, 2003, Sylwester *et al.*, 2005). This can be explained by $CD8^+$ T cells being primarily induced by cross priming (Tabi *et al.*, 2001). A recent study by Sylwester and colleagues demonstrated that 33 different open reading frames (ORF) from CMV were recognised by $CD8^+$ T cells from at least four out of a total of 33 CMV seropositive individuals (Sylwester *et al.*, 2005). In that study $CD8^+$ T cells recognised a median of 8 ORF with considerable interindividual heterogeneity (range 1 to 39). Given the vast number of $CD8^+$ T cell targets available and interindividual heterogeneity of T cell responses observed, it remains to be seen whether adoptive therapy may use T cells generated in a non-epitope specific manner in the future.

The highly selective nature of tetramer based cell selection makes it useful in the HSCT setting since it is likely associated with a low risk of GvHD development. Alternative cell selection methods, such as cytokine capture, however, provide the

advantage of isolating $CD4^+$ T cells along with $CD8^+$ T cells and were demonstrated to be feasible and effective in a clinical environment (Mackinnon *et al.*, 2007). This study suggests that the presence of at least 160 $CD4^+$ T cells/µl blood is necessary for efficient *in vivo* function of CMV specific $CD8^+$ T cells. Therefore therapeutic trials using a mix of T cell specificities comprising $CD4^+$ and $CD8^+$ T cells targeting CMV or even a mix of CMV and other viruses may be preferred in the immediate future before a wider range of clinical grade tetramers becomes available. Consistent with that recent studies moved on to assess cytotoxic T cell responses against multiple viral infections simultaneously using ELISpot based epitope matrices rather than tetramer based methods (Bihl *et al.*, 2005). In a recent clinical trial infusing $CD8^+$ T cells that were generated against CMV, Epstein-Barr virus (EBV) and adenovirus into 14 stem cell recipients demonstrated recovery of immunity to CMV and EBV in all patients, and to adenovirus in patients with evident adenovirus infection prior to $CD8^+$ T cell infusion (Leen *et al.*, 2006). Future studies may include other viruses that cause complications post HSCT including Human Herpes Virus (HHV) 6 and 7.

Despite these developments tetramer staining remains a valuable tool for highly specific selection and thereby therapeutic use of antigen specific T cells and for monitoring of those cells *ex vivo*. Cocktails containing a mixture of different tetramers should be clinically useful for the detection of antigen specific T cells. This study showed that they are also useful for clonal and phenotypic characterisation of selected cells that may aid the advancement of T cell immunotherapy. Adoptive therapy with cell populations selected with tetramers directly *ex vivo* avoids prolonged periods of expansion *in vitro* and the use of live viral antigen that may not be suitable for clinical applications. *In vivo* expansion and clinical efficiency of cells transferred in low numbers (median cell dosage: $8.6x10^3$/kg) after tetramer guided selection was demonstrated successfully (Cobbold *et al.*, 2005) and may confer an advantage over less specific approaches.

In the long run studies will need to address the question of how immunogenic $CD4^+$ and $CD8^+$ T cells targeting different CMV epitopes may be, to enable the development of a therapeutic approach comprising the epitope specific T cell populations that may provide maximal clinical benefit for patients. Recent advances in class II tetramer production comprising 16 different human HLA class II allotypes (Vollers and Stern, 2008) may enable tetramer guided selection of $CD4^+$ as well $CD8^+$ T cells for adoptive therapy of patients in the future.

An important role for $CD4^+$ T cells regarding their helper function for CD8 T^+ cells was demonstrated by many studies. Interestingly, cytotoxic subsets of CMV specific $CD4^+$ T cells that can lyse targets infected with CMV glycoprotein B (that accumulates in endosomes during host cell infection with CMV) were recently described (Hegde *et al.*, 2005). These cells may therefore have an important role in eliminating infected antigen presenting cells that express HLA class II. Furthermore a very recent report demonstrates high frequencies and a highly restricted TCR usage of these cells, which is unprecedented for $CD4^+$ T cells, along with phenotypic and functional properties similar to CMV specific $CD8^+$ T cells (Crompton *et al.*, 2008). This suggests that cytotoxic subsets of $CD4^+$ T cells might exert functions similar to those of $CD8^+$ T cells including the drive of immunosenescence in infected individuals. This awaits further studies to establish whether cytotoxic $CD4^+$ T cell subsets should be included in antiviral therapeutic strategies.

Patients receiving HSCT grafts from CMV seronegative donors would not be able to benefit from donor derived adoptive cell therapy. In these cases, vaccination of donors may be one way to make this treatment option available to a majority of patients.

In summary, findings from this study provide a deeper understanding of CMV specific $CD8^+$ T cells with previously less well studied target specificities in HSCT patients. Numbers of these cells that inversely correlated with the ability to detect CMV reactivation were shown for the first time. A significant difference in TCR diversity between the cells of different specificity was presented. The presence of most of these CMV specific $CD8^+$ T cells in a majority of patients underlines their clinical relevance. Thus the findings presented here may further the prospects of the development of new prophylactic and therapeutic anti-CMV treatments in the future.

CHAPTER 6 APPENDIX

6-1 Appendix for section 2-11.1

pET-3a-d Vectors

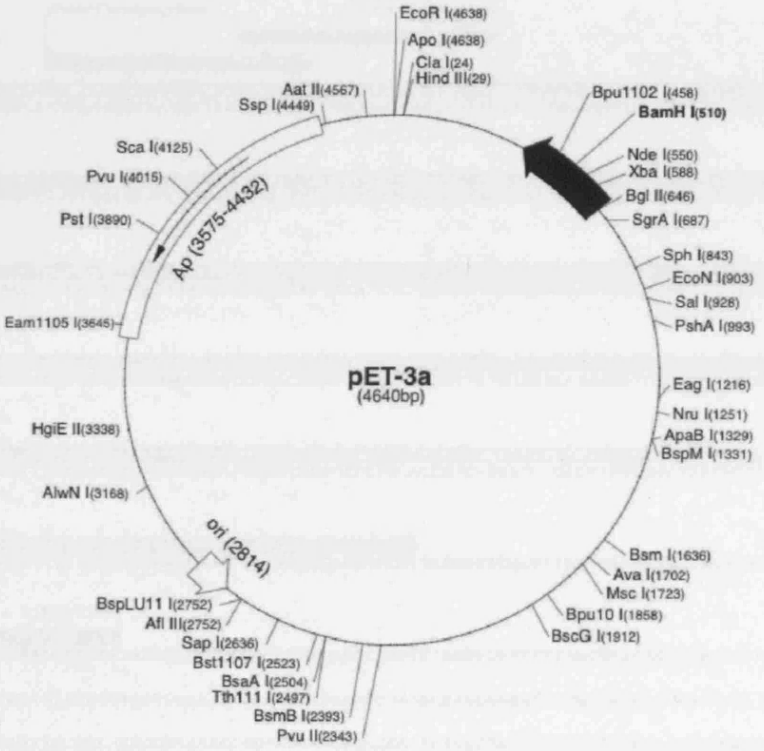
TB026 12/98

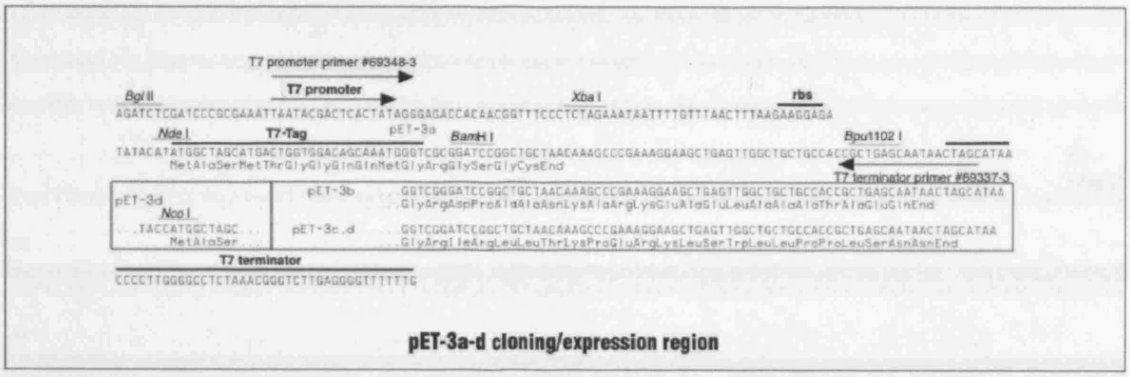
	Cat. No.
pET-3a DNA	69418-3
pET-3b DNA	69419-3
pET-3c DNA	69420-3
pET-3d DNA	69421-3

The pET-3a-d vectors carry an N-terminal T7•Tag® sequence and *Bam*H I cloning site. These vectors are the precursors to many pET family vectors; the pET-23a-d(+) series corresponds to pET-3a-d but incorporates several additional features. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

pET-3a sequence landmarks	
T7 promoter	615-631
T7 transcription start	614
T7•Tag coding sequence	519-551
T7 terminator	404-450
pBR322 origin	2814
bla coding sequence	3575-4432

The maps for pET-3b, pET-3c and pET-3d are the same as pET-3a (shown) with the following exceptions: pET-3b is a 4639bp plasmid; subtract 1bp from each site beyond *Bam*H I at 510. pET-3c is a 4638bp plasmid; subtract 2bp from each site beyond *Bam*H I at 510. pET-3d is a 4637bp plasmid; the *Bam*H I site is in the same reading frame as in pET-3c. An *Nco* I site is substituted for the *Nde* I site with a net 1bp deletion at position 550 of pET-3c. As a result, *Nco* I cuts pET-3d at 546. For the rest of the sites, subtract 3bp from each site beyond position 551 in pET-3a. *Nde* I does not cut pET-3d.

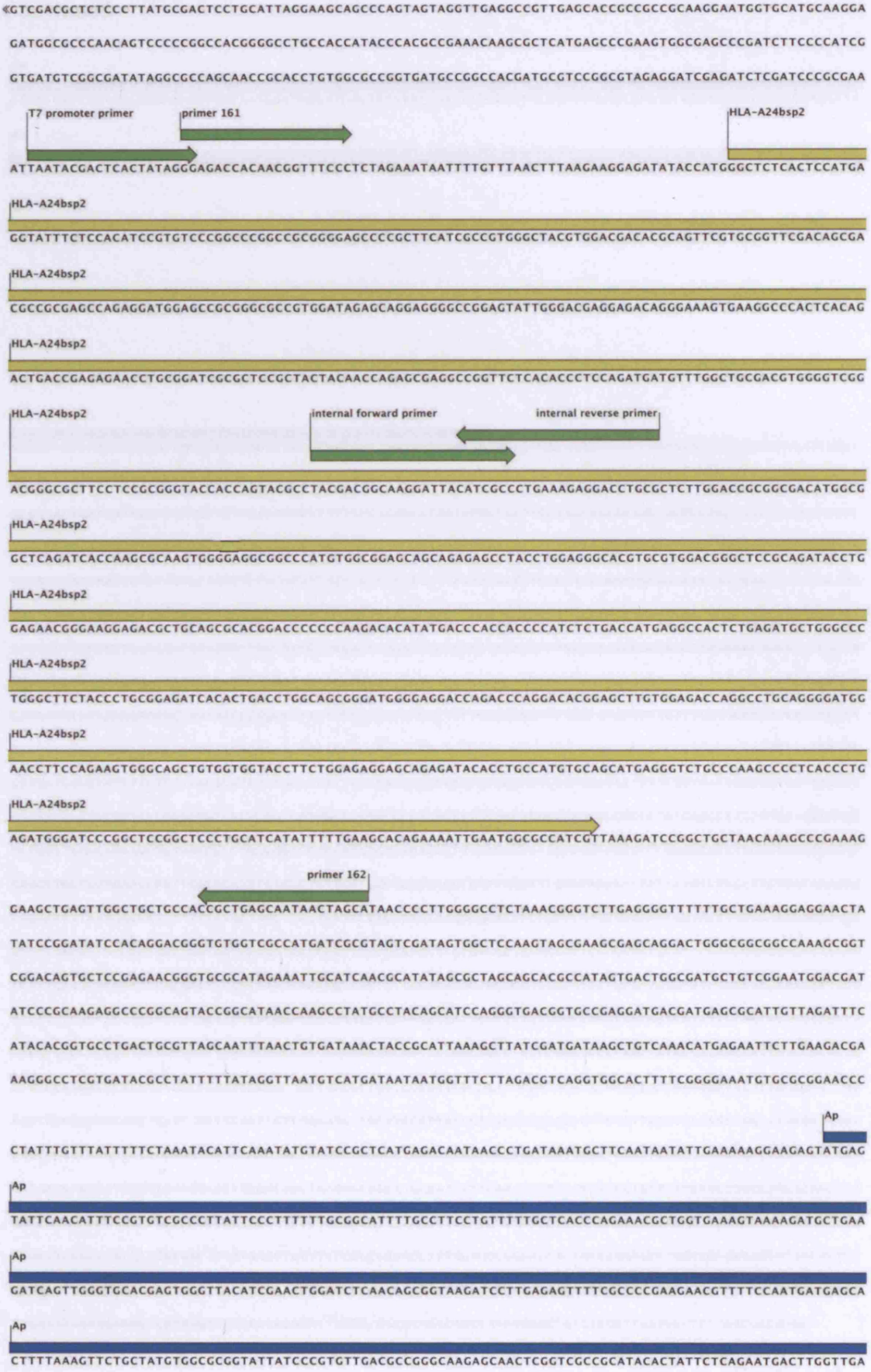




pET-3a-d cloning/expression region

Figure 6-1 Map of the original pET3d vector

This figure is an amendment of Figure 2-16. It shows the map of the original pET3d vector (Novagen) used for cloning of HLA-A24 (adopted from http://www.merckbiosciences.co.uk/Products).


GTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGA
GATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCG
GTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAA
T7 promoter primer
primer 161
HLA-A24bsp2
ATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCTCTCACTCCATGA
HLA-A24bsp2
GGTATTTCTCCACATCCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCATCGCCGTGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGA
HLA-A24bsp2
CGCCGCGAGCCAGAGGATGGAGCCGCGGGCGCCGTGGATAGAGCAGGAGGGGCCGGAGTATTGGGACGAGGAGACAGGGAAAGTGAAGGCCCACTCACAG
HLA-A24bsp2
ACTGACCGAGAGAACCTGCGGATCGCGCTCCGCTACTACAACCAGAGCGAGGCCGGTTCTCACACCCTCCAGATGATGTTTGGCTGCGACGTGGGGTCGG
HLA-A24bsp2
internal forward primer
internal reverse primer
ACGGGCGCTTCCTCCGCGGGTACCACCAGTACGCCTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCTGCGCTCTTGGACCGCGGCGACATGGCG
HLA-A24bsp2
GCTCAGATCACCAAGCGCAAGTGGGGAGGCGGCCCATGTGGCGGAGCAGCAGAGAGCCTACCTGGAGGGCACGTGCGTGGACGGGCTCCGCAGATACCTG
HLA-A24bsp2
GAGAACGGGAAGGAGACGCTGCAGCGCACGGACCCCCCCAAGACACATATGACCCACCACCCCATCTCTGACCATGAGGCCACTCTGAGATGCTGGGCCC
HLA-A24bsp2
TGGGCTTCTACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGGGAGGACCAGACCCAGGACACGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGG
HLA-A24bsp2
AACCTTCCAGAAGTGGGCAGCTGTGGTGGTACCTTCTGGAGAGGAGCAGAGATACACCTGCCATGTGCAGCATGAGGGTCTGCCCAAGCCCCTCACCCTG
HLA-A24bsp2
AGATGGGATCCCGGCTCCGGCTCCCTGCATCATATTTTTGAAGCACAGAAAATTGAATGGCGCCATCGTTAAAGATCCGGCTGCTAACAAAGCCCGAAAG
primer 162
GAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTA
TATCCGGATATCCACAGGACGGGTGTGGTCGCCATGATCGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGT
CGGACAGTGCTCCGAGAACGGGTGCGCATAGAAATTGCATCAACGCATATAGCGCTAGCAGCACGCCATAGTGACTGGCGATGCTGTCGGAATGGACGAT
ATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAGCCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTC
ATACACGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTCAAACATGAGAATTCTTGAAGACGA
AAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCC
Ap
CTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAG
Ap
TATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAA
Ap
GATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCA
Ap
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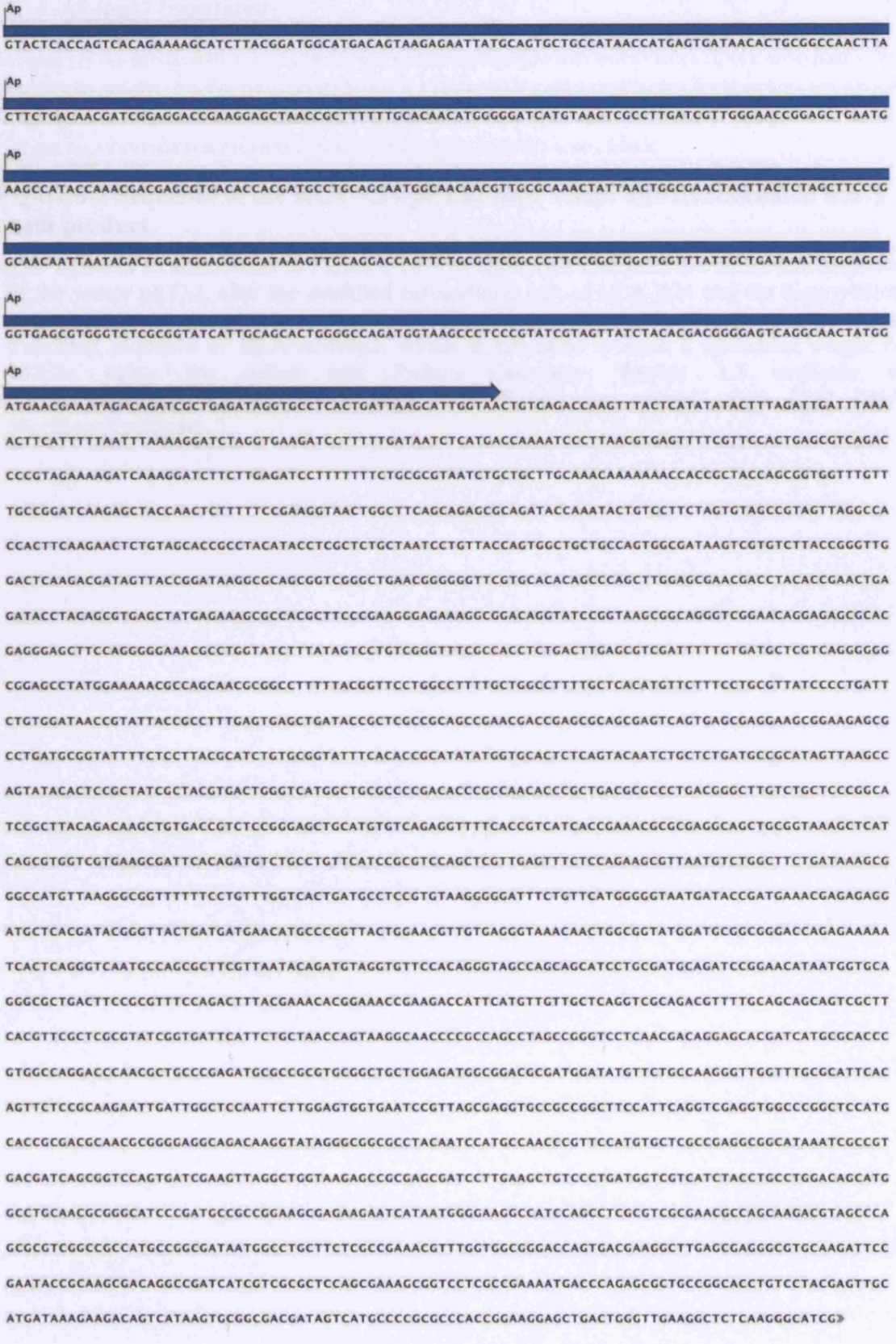
Continued

Continued



Continued

Continued

HLA-A24*bsp2* translated:

GSHSMRYFSTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDEETGKVKAHSQTDRENLRI
ALRYYNQSEAGSHTLQMMFGCDVGSDGRFLRGYHQYAYDGKDYIALKEDLRSWTAADMAAQITKRKWEAAHVAEQQR
AYLEGTCVDGLRRYLENGKETLQRTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAG
DGTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWDPGSGSLHHIFEAQKIEWRHR

Figure 6-2 Sequence of the HLA-A24-pET3d-bsp2 vector and its translated heavy chain product

This figure is an amendment to Figure 2-16. The upper part illustrates the nucleotide sequence of the vector pET3d, after the modified extracellular part of HLA-A24 and the biotinylation target bsp2 were cloned into it (Ap: ampicillin resistance gene). The lower part shows the translated sequence of HLA-A24bsp2, which is estimated to have a molecular weight of 34 kDa using the online tool Protein Calculator Version 3.3 available at http://scripps.edu/~cdputnam/protcalc.html. This figure was created using CLC DNA Workbench software.

6-2 Appendix for section 3-2.1.3

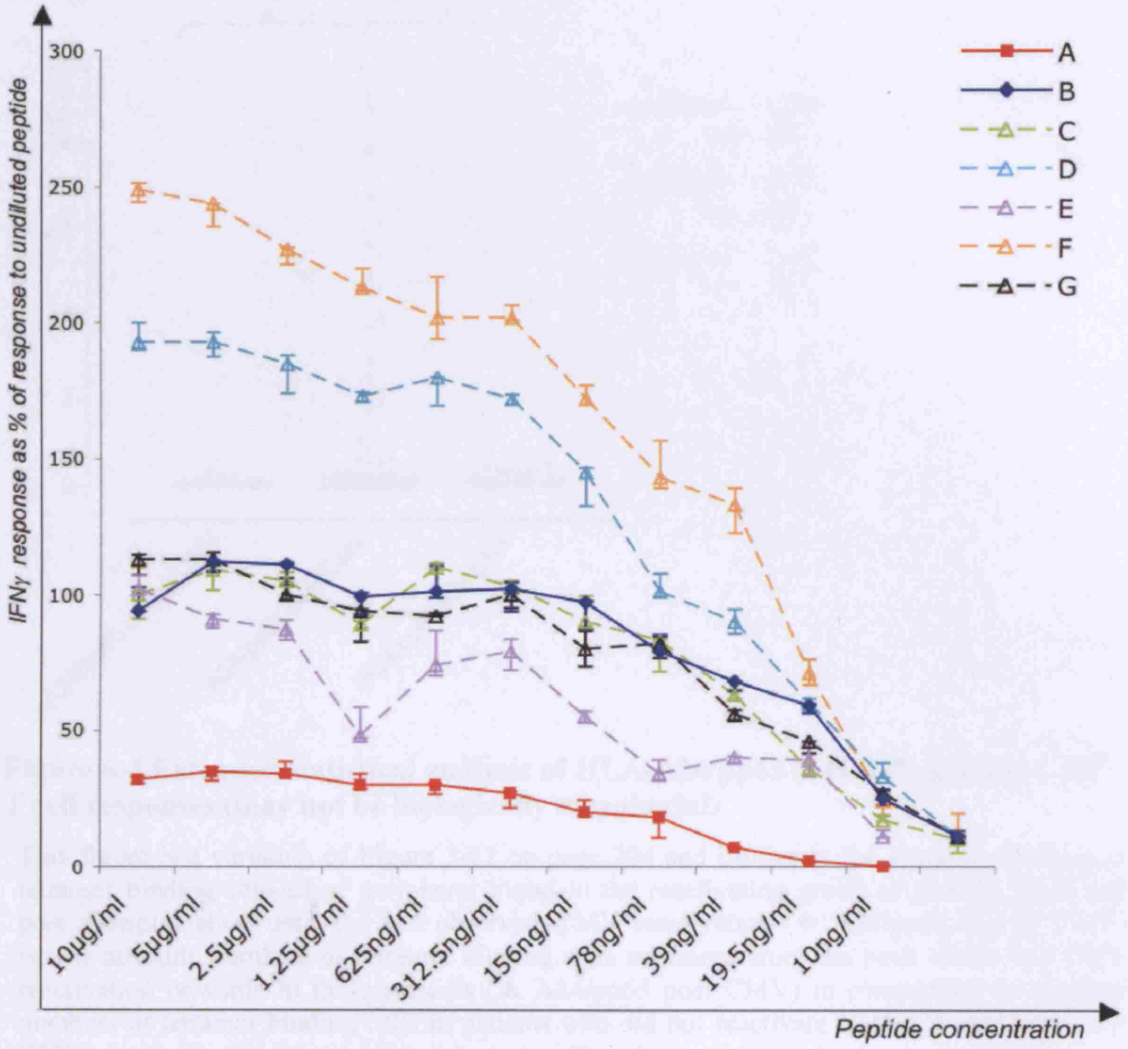


Figure 6-3 Variation of illustration: IFNγ release of cells in response to serial dilutions of CMV peptide pp65 (123-131)

This figure is a variation of Figure 3-3. It shows IFNγ release of PBMC in responses to 1:2 dilutions of CMV pp65 (123-131) peptide that was measured in triplicate in two samples from patient 7 (solid lines, A: sample taken at day 77, B: sample taken at day 818 post transplantation) and five samples from other patients (dashed lines, C: patient 30b at 763 days post transplantation, D: patient 31 at 700 days post transplantation, E: patient 6 at 86 days post transplantation, F: patient 9 at 367 days post transplantation and G: patient 32 at 483 days post transplantation). In comparison to Figure 3-3, this illustration shows the original median SFU values above unstimulated controls from triplicate measurements with errors represented as the difference to 25 and 75 percentiles.

6-3 Appendix for section 3-2.2.3

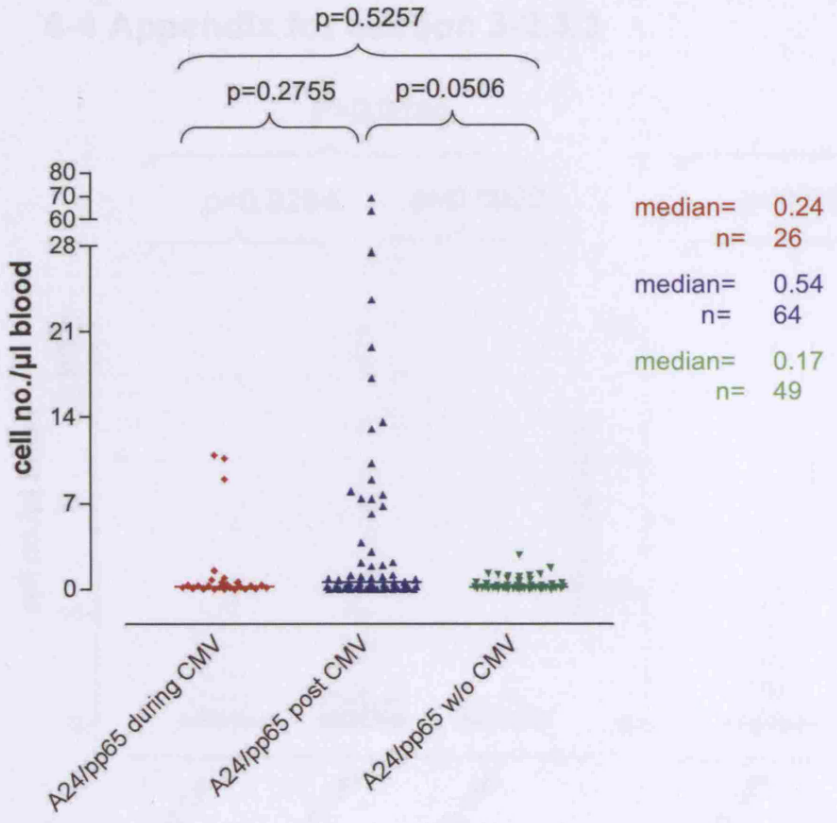


Figure 6-4 Extended statistical analysis of HLA-A24/pp65 (341-349) specific CD8$^+$ T cell responses (may not be biologically meaningful)

This figure is a variation of Figure 3-11 on page 204 and illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in the reactivating group of patients measured post transplantation until the last observed CMV reactivation (◆ A24/pp65 during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in these patients (▲ A24/pp65 post CMV) in comparison to absolute numbers of tetramer binding cells in patients who did not reactivate CMV (▼ A24/pp65 w/o CMV). In contrast to Figure 3-11, it includes all patients of the study group except patient 20 (CMV-/+ without primary infection), which may not be biologically meaningful. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values ($p<0.02$ regarded significant) indicated in the graph.

6-4 Appendix for section 3-2.3.3

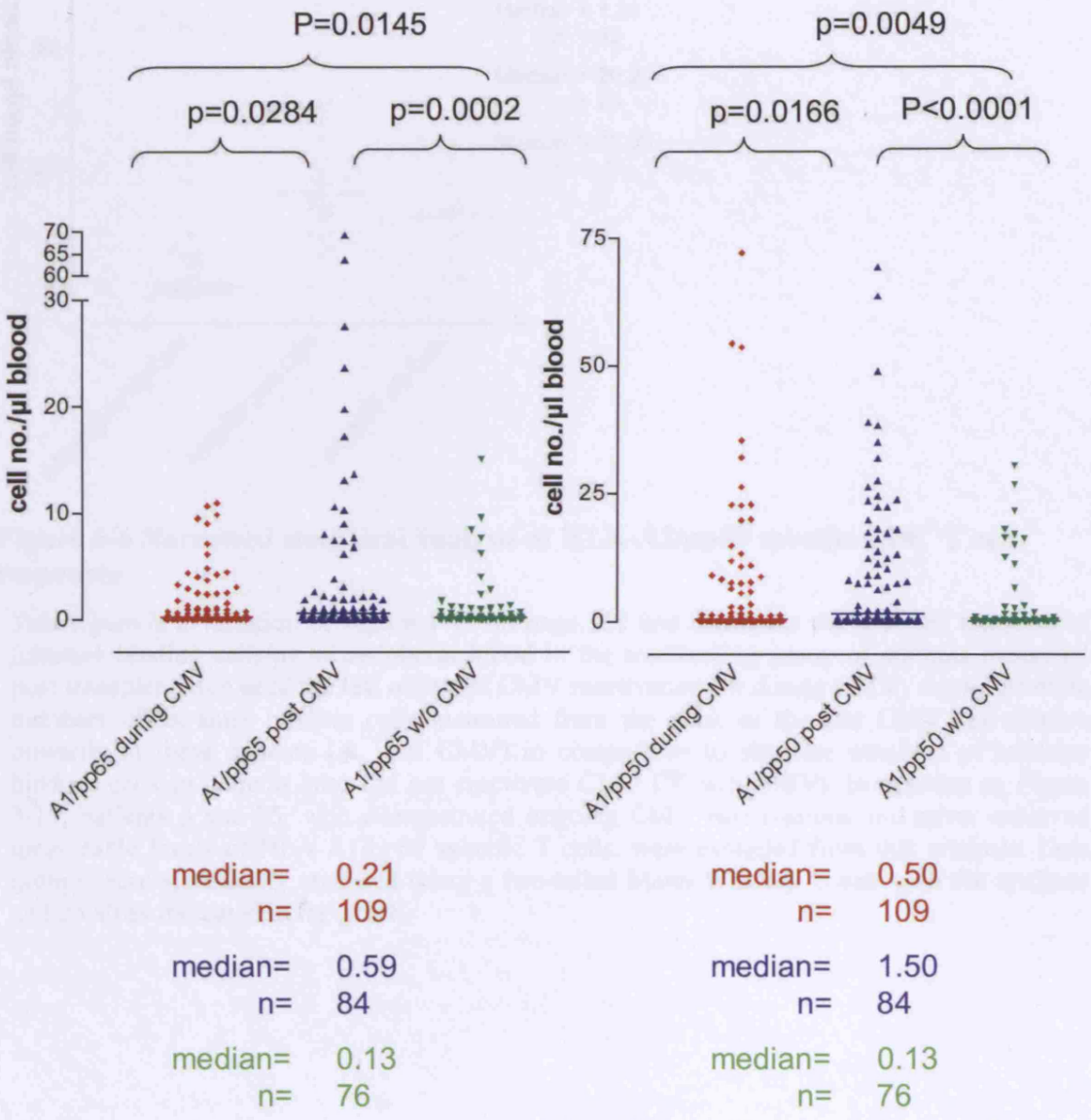


Figure 6-5 Extended statistical analysis of HLA-A1/CMV specific CD8$^+$ T cell responses (may not be biologically meaningful)

This figure is a variation of Figure 3-19 on page 222 and illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in the reactivating group of patients measured post transplantation until the last observed CMV reactivation (◆ during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in these patients (▲ post CMV) in comparison to absolute numbers of tetramer binding cells in patients who did not reactivate CMV (▼ w/o CMV). In contrast to Figure 3-19, all patients of this study group, except patients 27 and 28 (CMV-/+ without primary infection) were included in this analysis, which may not be biologically meaningful. The comparison is extended to HLA-A1/pp50 and HLA-A1/pp65 specific CD8$^+$ T cell responses. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values indicated in the graph.

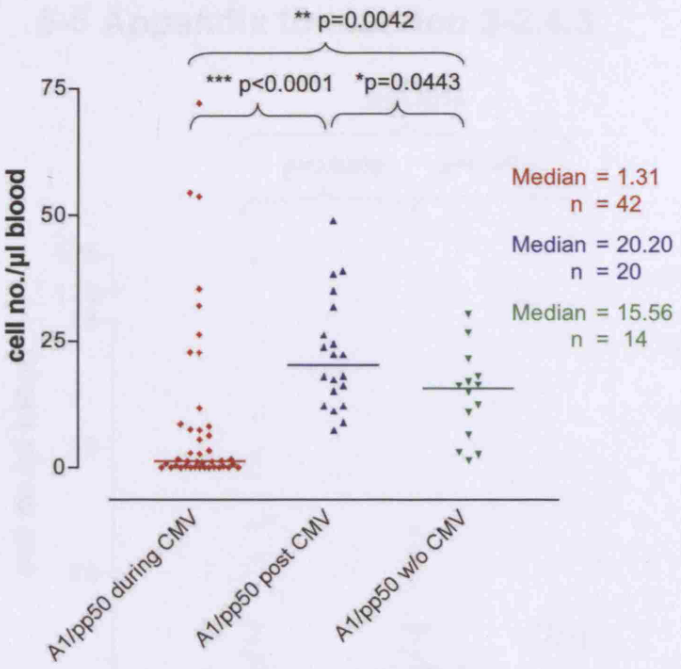


Figure 6-6 Narrowed statistical analysis of HLA-A1/pp50 specific CD8$^+$ T cell responses

This figure is a variation of Figure 3-19 on page 222 and illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in the reactivating group of patients measured post transplantation until the last observed CMV reactivation (◆ during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in these patients (▲ post CMV) in comparison to absolute numbers of tetramer binding cells in patients who did not reactivate CMV (▼ w/o CMV). In contrast to Figure 3-19, patients 3 and 25, who demonstrated ongoing CMV reactivations and never achieved measurable levels of HLA-A1/pp50 specific T cells, were excluded from this analysis. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values indicated in the graph.

6-5 Appendix for section 3-2.4.3

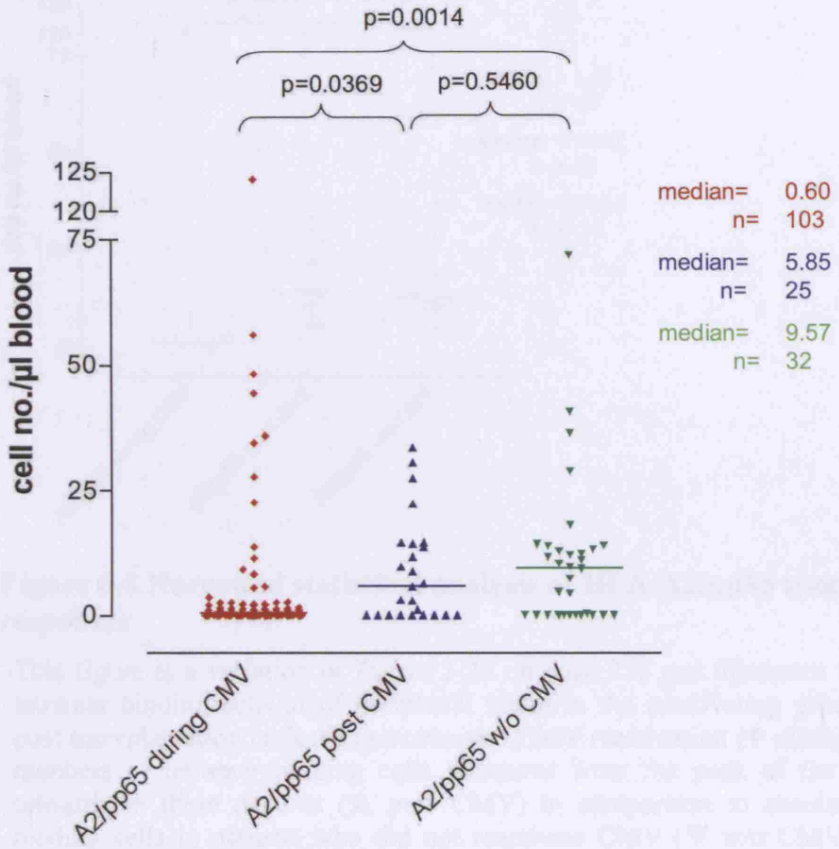


Figure 6-7 Extended statistical analysis of HLA-A2/pp65 specific CD8$^+$ T cell responses (may not be biologically meaningful)

This figure is a variation of Figure 3-25 on page 235 and illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in the reactivating group of patients measured post transplantation until the last observed CMV reactivation (◆ during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in these patients (▲ post CMV) in comparison to absolute numbers of tetramer binding cells in patients who did not reactivate CMV (▼ w/o CMV). In contrast to Figure 3-25, all patients of the study group (including patients 1, 2, and 26 suspected to be protected by another response and patients 24 and 29 with low CD4$^+$ T cells) were included in this analysis, which may not be biologically meaningful. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values indicated in the graph.

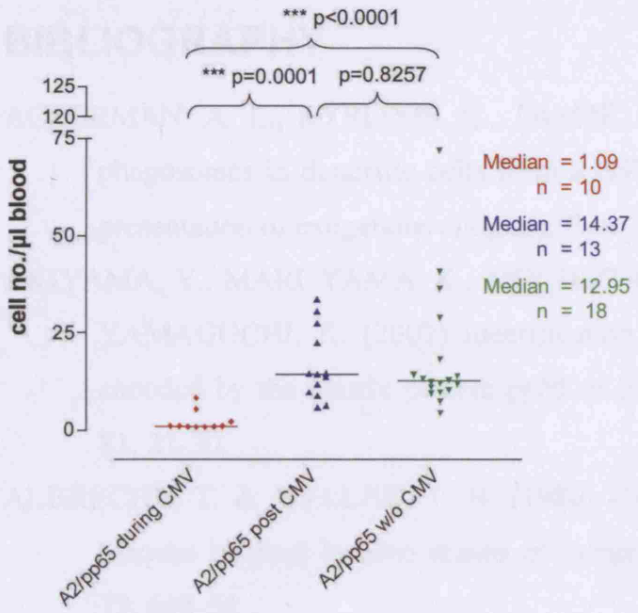


Figure 6-8 Narrowed statistical analysis of HLA-A2/pp65 specific CD8$^+$ T cell responses

This figure is a variation of Figure 3-25 on page 235 and illustrates the absolute numbers of tetramer binding cells/µl of peripheral blood in the reactivating group of patients measured post transplantation until the last observed CMV reactivation (◆ during CMV) *versus* absolute numbers of tetramer binding cells measured from the peak of the last CMV reactivation onwards in these patients (▲ post CMV) in comparison to absolute numbers of tetramer binding cells in patients who did not reactivate CMV (▼ w/o CMV). In contrast to Figure 3-25, patient 30, who demonstrated ongoing CMV reactivations and never achieved measurable levels of HLA-A2/pp65 specific T cells, was excluded from this analysis. Data groups were statistically analysed using a two-tailed Mann-Whitney U test with the medians and p values indicated in the graph.

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